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(57) Abstract

The present invention relates generally to a plant regulatory gene and derivatives and homologues thereof. More particularly, the present invention provides a nucleic acid molecule comprising a sequence of nucleotides or complementary sequence of nucleotides, the expression of which, modulates or otherwise facilitates activity of a cytochrome P450 protein in plant cells and tissues including petals, flowers, stems, leaves and seeds. Even more particularly, the nucleic acid molecule of the present invention encodes a cytochrome b_5 or a mutant, part, fragment, portion, functional and/or structural equivalent or homologue thereof or agonist or antagonist thereof involved in modulating or otherwise facilitating activity of a dihydrokaempferol (DHK) hydroxylating enzyme such as but not limited to flavonoid 3', 5'-hydroxylase. The present invention further provides transgenic plants or parts thereof or cells of transgenic plants as well as cut or severed flowers or stems from transgenic plants.

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A PLANT REGULATORY GENE

The present invention relates generally to a plant regulatory gene and derivatives and homologues thereof. More particularly, the present invention provides a nucleic acid molecule comprising a sequence of nucleotides or complementary sequence of nucleotides, the expression of which, modulates or otherwise facilitates activity of a cytochrome P450 protein in plant cells and tissues including petals, flowers, stems, leaves and seeds. Even more particularly, the nucleic acid molecule of the present invention encodes a cytochrome b_5 or a mutant, part, fragment, portion, functional and/or structural equivalent or homologue thereof or agonist or antagonist thereof involved in modulating or otherwise facilitating activity of a dihydrokaempferol (DHK) hydroxylating enzyme such as but not limited to flavonoid 3′, 5′-hydroxylase. The present invention further provides transgenic plants or parts thereof or cells of transgenic plants as well as cut or severed flowers or stems from transgenic plants.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The subject specification contains nucleotide and amino acid sequence information prepared using the programme PatentIn Version 2.0, presented herein after the bibliography. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (eg. <400>1, <400>2, etc).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

The flower industry strives to develop new and different varieties of flowering plants. Altering flower colour has become a particularly important aim in the research and development undertaken by or on behalf of the flower industry.

Classical breeding techniques have been successfully employed to develop new flowering varieties. However, the generation of plants with desired traits is constrained by, for example, the species' gene pool, a development process which is time consuming and a frequently low success rate. The rapidly increasing sophistication of genetic engineering techniques offers a great opportunity to develop new varieties of plants without some or all of the above constraints.

One important area of flowering plant development is the generation of plants with altered flower colour. Flower colour is predominantly due to two different pigments: flavonoids and carotenoids. The flavonoids contribute to a range of colours from yellow to red to blue. Carotenoids impart an orange or yellow tinge. Particularly important flavonoid molecules include the anthocyanins which are glycosylated derivatives of cyanidin, delphinidin, petunidin, peonidin, malvidin and pelargonidin.

The biosynthetic pathway for flavonoid pigments (hereinafter referred to as the "flavonoid

pathway") is now well established (1). The essence of the pathway is a condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA which is catalysed by chalcone synthase. The product of this reaction, 2′, 4, 4′, 6′-tetrahydroxychalcone, is generally rapidly isomerised to produce naringenin by chalcone flavanone isomerase. Naringenin is subsequently hydroxylated at the 3′ position of the central ring by flavanone 3-hydroxylase to produce dihydrokaempferol (DHK).

The B-ring of the DHK can be hydroxylated at either the 3′ or both 3′ and 5′ positions to produce dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. Two key enzymes involved in this pathway are flavonoid 3′-hydroxylase (hereinafter referred to as "F3′H") and flavonoid 3′, 5′-hydroxylase (hereinafter referred to as "F3′5′H").

F3′H acts on DHK to produce DHQ and on naringenin to produce eriodicytyol. F3′5′H is a broad spectrum enzyme catalysing hydroxylation of naringenin and DHK in the 3′ and 5′ positions, in both instances producing pentahydroxyflavanone and DHM, respectively, as well as catalysing hydroxylation of DHQ in the 5′ position. The essence of these catalysed reactions is shown in Figure 1 (1).

The ability to control F3′H and F3′5′H activity in plants provides a means to manipulate flower colour. This is successfully described, for example, in International Patent Application Nos. PCT/AU92/00334, PCT/AU93/00127 and PCT/AU94/00265. Levels of F3′H and F3′5′H can be manipulated using genetic means such as altering promoter strength, using anti-sense and ribozyme technologies and through co-suppression. It is important, however, to fully elucidate the endogenous regulatory mechanisms for these and other flavonoid pathway enzymes. Such knowledge can lead to even greater modulation of enzyme activity, especially in plants where expression of, for example, a F3′5′H gene is low.

In work leading up to the present invention, the inventors sought to identify genes involved in the regulation of anthocyanin modification. In accordance with the present invention, the inventors have now identified a molecule which modulates or other

otherwise facilitates activity of a flower cytochrome P450.

The present invention is predicated in part on the isolation of a genetic sequence which encodes a polypeptide which represents a novel class of plant cytochrome b_5 (Cyt b_5) molecules. The genetic sequence of the present invention is referred to and exemplified herein by *"difF"* which includes a sequence of nucleotides or complementary sequence of nucleotides which encodes the Cyt b_5, i.e., DIF-F, or a mutant, part, fragment, portion thereof or a functional and/or structural equivalent or homologue thereof. For convenience, all such mutants, parts, fragments and portions are referred to herein as a "derivative" or "derivatives". A "derivative" includes mutants, parts, fragments, portions, variants and fusions of the Cyt b_5 protein or corresponding *difF* gene as well as single or multiple nucleotide substitutions, additions and/or deletions of *difF*. A "derivative" may also include an agonist or antagonist of Cyt b_5. The term *"difF"* includes a genomic DNA isolate as well as a cDNA molecule or a chemically prepared molecule generated by the stepwise addition of nucleotides or chemical equivalents thereof. The term *"difF"* is generically used herein to encompass any molecule encoding a Cyt b_5 or a derivative or homologue thereof and which modulates or otherwise facilitates activity of a Cyt p450 such as but not limited to Cyt P450's involved or otherwise associated with the hydroxylation of a flavonoid compound. An example, the Cyt b_5 or derivative might impact on the activity of a Cyt P450 or may indirectly act *via* a reductase such as NADPH cytochrome P450 reductase.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a Cyt b_5 molecule or a derivative, homologue or functional equivalent thereof.

More particularly, the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a Cyt b_5 molecule or a derivative, homologue or functional equivalent thereof wherein said Cyt b_5 modulates or otherwise facilitates activity of a Cyt P450.

Even more particularly, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a Cyt b_5 molecule or a derivative, homologue or functional equivalent thereof wherein said Cyt b_5 modulates or otherwise facilitates activity of a Cyt P450 wherein the Cyt P450 comprises the amino acid sequence (F/P/W/L/Y)(G/S/C/N/H/E)X(G/D/A/E)X (R/H/S/K/T) XCX_a(G/A) wherein X is any amino acid and X_a is selected from V, M, A, L, I, P, F or T and wherein the residues in parentheses represent alternatives of a single position.

Still more particularly, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a Cyt b_5 molecule or a derivative, homologue or functional equivalent thereof wherein said Cyt b_5 modulates or otherwise facilitates activity of a Cyt P450 enzyme comprising the amino acid sequence (F/P/W/L/Y)(G/S/C/N/H/E)X(G/D/A/E)X (R/H/S/K/T) XCX_a(G/A) wherein X is any amino acid and X_a is selected from V, M, A, L, I, P, F or T and wherein the residues in parentheses represent alternatives of a single position and which Cyt P450 is involved in or otherwise associated with the direct or indirect hydroxylation of a flavonoid compound.

The preferred Cyt b_5 of the present invention is DIF-F which modulates or otherwise facilitates activity of F3′5′H. However, the present invention extends to other Cyt b_5 molecules which facilitate activity of any Cyt P450 capable of directly or indirectly hydroxylating a flavonoid compound. The present invention is hereinafter described with reference to a Cyt b_5 and its activity on F3′5′H but this is done with the understanding that the present invention extends to any member of the Cyt b_5 family especially those involved in the direct or indirect hydroxylation of a flavonoid compound. Examples of other enzymes which are modified or otherwise facilitated by Cyt b_5 include but are not limited to F3′H.

Although not intending to limit the invention to any one theory or mode of action, Cyt b_5 molecules are believed to interact with cytochrome P450 *via* electrostatic interactions.

These interactions are determined by the primary as well as the tertiary structure of the proteins. An amino acid sequence alignment of petunia Cyt b_5 with other plant Cyt b_5 sequences reveals two regions in the petunia Cyt b_5 sequence that have insertions of six (SELELN) and nine (EDPKPKYLT) amino acids in length. Furthermore, petunia Cyt b_5 protein has a net positive charge while other plant cytochrome b_5 proteins have a net negative charge. These insertions, which result in a longer petunia Cyt b_5 as well as a change in its charge distribution, may influence the interaction of petunia Cyt b_5 with cytochrome P450 proteins such as petunia F3'5'H.

The *difF* of the present invention is considered to reside on a separate phylogenetic branch to known Cyt b_5 genes. The preferred novel Cyt b_5 molecule encoded by *difF* comprises the amino acid sequence YKASDDSELELNLVTDSIKEPN or an amino acid sequence having at least about 70% similarity thereto. Even more preferably, the Cyt b_5 of the present invention comprises the amino acid sequence:

$[X_1\ X_2\ \ldots\ldots\ldots X_n]$ KE $[X_1'\ X_2'\ \ldots\ldots\ X_{n1}']$F $[X_1''\ X_2''\ \ldots\ldots\ldots X''_{n2}]$
YKASDDSELELNLVTDSIKEPNDSIKEPN $[X_1'''\ X_2'''\ \ldots\ldots\ldots X'''_{n3}]$ EDPKPYLTFVEY

wherein $[X_1\ X_2\ \ldots\ldots\ldots X_n]$, $[X_1'\ X_2'\ \ldots\ldots\ldots X'_{n1}{}']$, $[X_1''\ X_2''\ \ldots\ldots\ldots X''_{n2}]$ and $[X_1'''\ X_2'''\ \ldots\ldots\ldots X'''_{n3}{}''']$ are sequences of any amino acid residues up to n_1, n_2 and n_3 amino acid residues in length wherein n, n_1, n_2' and n_3' may be the same or different and each is from about 1 to about 200.

In a preferred aspect of the present invention, there is provided an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a Cyt b_5 molecule or a derivative, homologue or functional equivalent thereof wherein said Cyt b_5 molecule modulates or otherwise facilitates activity of F3'5'H or a derivative, homologue or functional equivalent thereof.

Preferably, the Cyt b_5 is expressed substantially exclusively in the flower although any Cyt b_5 is contemplated by the present invention provided it modulates or otherwise facilitates

activity of a Cyt P450 molecule. In a particularly preferred embodiment, the Cyt b_5 modulates or otherwise facilitates activity of F3'5'H in flowers.

According to this preferred embodiment, there is provided an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a Cyt b_5 molecule or a derivative, homologue or functional equivalent thereof wherein said Cyt b_5 modulates or otherwise facilitates activity of F3'5'H or a derivative, homologue or functional equivalent thereof substantially exclusively in flowers.

In one particular embodiment, the Cyt b_5 modulates or otherwise facilitates activity of F3'5'H in flowers. In another embodiment, over expression of *difF* may also enhance F3'H activity.

Although not intending to limit the present invention to any one theory or mode of action the Cyt b_5 of the present invention may act at the level of activity of hydroxylating enzyme (e.g. F3'5'H), at the level of gene transcription (e.g. a transcription regulator) or at the level of translation. The Cyt b_5 may also act alone or in association with another molecule. For example, the Cyt b_5 may form a complex with another molecule, e.g. a reductase, and the Cyt b_5 complex may then act on the hydroxylating enzyme or its genetic sequence. Alternatively, the Cyt b_5 molecule may require the interaction of another molecule at the level of hydroxylating enzyme or its genetic sequence. In particular, one of Cyt b_5 or another molecule may interact with the flavonoid hydroxylating enzyme or genetic sequence encoding same and simultaneously or sequentially, the other of the Cyt b_5 or another molecule may also interact with the flavonoid hydroxylating enzyme or its genetic sequence. The effect(s) of the Cyt b_5 on modulating or otherwise facilitating activity of the hydroxylating enzyme may require interaction of both Cyt b_5 and the other molecule.

The preferred Cyt b_5 is DIF-F and comprises the amino acid sequence substantially as set forth in <400>2.

Accordingly, another aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides or complementary sequence of nucleotides encoding a Cyt b_5 protein or a derivative, homologue or functional equivalent thereof having the amino acid sequence substantially as set forth in <400>2 or an amino acid sequence having at least about 30% similarity thereto and which Cyt b_5 or derivative, homologue or functional equivalent modulates or otherwise facilitates activity of a flavonoid hydroxylating enzyme such as but not limited to F3'5'H.

The percentage amino acid similarity may be at least about 40%, or at least about 50%, or at least about 60%, or least about 70%, or at least about 80%, or at least about 90-95% or greater to the amino acid sequence set forth in <400>2.

Yet another aspect of the present invention contemplates an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary sequence encoding a Cyt b_5 or a derivative, homologue or functional equivalent thereof wherein the nucleotide sequence is substantially as set forth in <400>1 or a nucleotide sequence having at least 30% similarity thereto or is a nucleotide sequence capable of hybridizing to <400>1 under low stringency conditions at 42°C.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity. Any number of programs are available to compare nucleotide and amino acid sequences. Preferred programs have regard to an appropriate alignment. One such program is Gap which considers all possible alignment and gap positions and creates an alignment with the largest number of matched bases and the fewest gaps. Gap uses the

alignment method of Needleman and Wunsch (18). Gap reads a scoring matrix that contains values for every possible GCG symbol match. GAP is available on ANGIS (Australian National Genomic Information Service) at website http://mel1.angis.org.au.

Preferably, the percentage identity is considered rather than percentage similarity. The term "identity" is used in its broadest sense to include the exact nucleotide or amino acid matches having regard to an appropriate alignment using a standard algorithm. Convenient algorithm in this regard include the Geneworks program (Intelligenetics).

The percentage nucleotide similarity may be at least about 40%, or at least about 50%, or at least about 60%, or least about 70%, or at least about 80%, or at least about 90-95% or greater to the nucleotide sequence set forth in <400>1.

Reference herein to a low stringency at 42°C includes and encompasses from at least about 0% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions.

The present invention further provides isolated naturally occurring and recombinant or chemically synthetic forms of DIF-F or other related Cyt b_5 molecules or their derivatives, homologues or functional equivalents thereof. The molecules may be in isolated form or when present in a plant cell. The present invention further extends to antibodies to DIF-F and related Cyt b_5 molecules or their derivatives, homologues or functional equivalents. Such antibodies are useful in the immunological detection and/or analysis of plants. The present invention also extends to agonists and antagonists of the Cyt b_5 molecules.

Conveniently, where appropriate, such agonists and antagonists come under the terms "derivative" or "derivatives".

It is proposed in accordance with the present invention that a functional Cyt b_5 (e.g. DIF-F encoded by *difF)* is required for activity of a flavonoid hydroxylating enzyme such as F3'5'H. The term "activity" includes full activity or enhanced, heightened or otherwise facilitated activity. Accordingly, it is further proposed that genetic constructs carrying a nucleotide sequence encoding a flavonoid hydroxylating enzyme such as but not limited to F3'5'H either contain a *difF* or a functional derivative, homologue or equivalent thereof or are used in conjunction with a genetic construct carrying a *difF* or its derivative, homologue or functional equivalent thereof.

Accordingly, another aspect of the present invention contemplates a genetic construct optionally further comprising cistrons encoding one or both of a Cyt P450 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent of homologue thereof; and/or a reductase or other associated protein.

A single cistron comprises a coding sequence of a particular protein under the control of a promoter sequence. The coding sequence is said to be operably linked to the promoter sequence multiple cistrons may each be under the control of a promoter.

Generally, the Cyt b_5 modulates or otherwise facilitates activity of the Cyt P450 enclosed by the same genetic construct or a Cyt P450 on another genetic construct or encoded by the genome of a host cell.

An "associated protein" is a protein which catalyses the transfer of electrons from, for example, a co-enzyme to a prosthetic haem group on reductase. An associated protein may also have a role in facilitating interaction between a Cyt P450 and a reductase.

In another embodiment of the present invention, there is provided a genetic construct carrying *difF* or its functional derivative, homologue or equivalent thereof said genetic

construct further comprising a gene for a flavonoid hydroxylating enzyme such as but not limited to a gene encoding a F3′5′H or a functional derivative, homologue or equivalent thereof.

Another aspect of the present invention provides a transgenic plant or part thereof or cells therefrom comprising genetic material encoding a Cyt b_5 molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent or homologue thereof.

A "part" of a plant includes plant cells and tissues such as petals, flowers, stems, leaves and seeds. Parts of plants include cut or severed flowers.

Yet another aspect of the present invention contemplates a transgenic plant or parts thereof or cells of a transgenic plant, said plant or plant cells comprising genetic material corresponding to *difF* or a functional derivative, homologue or equivalent thereof and optionally a gene encoding an F3′5′H or its derivative, homologue or equivalent.

As stated above, reference to cells of a transgenic plant includes reference to tissues and organs of a plant. Reference to "parts" of a transgenic plant includes flowers (e.g. cut flowers) or flowering plants such as petals.

The present invention also extends to other cells containing or carrying the genetic constructs herein described. Such other cells include yeast cells and bacterial cells.

In petunia, the alleles encoding F3′5′H are referred to as *"hf1"* and *"hf2"*. It is proposed, in accordance with the present invention, that *difF* and/or the product of *difF* (DIF-F) have a role in facilitating activity of F3′5′H or expression of a gene encoding F3′5′H or its derivatives or homologues or other flavonoid pathway enzymes such as F3′H or its genetic sequences. The present invention extends, however, to the effects of *difF* or its product DIF-F or other related Cyt b_5 molecule on any gene or allele encoding an F3′5′H or a functional derivative, homologue or equivalent thereof. The present invention also

extends, in one particular embodiment, to the effect of *difF* or DIF-F facilitating or otherwise influencing expression of *hf1* and *hf2*.

Yet another aspect of the present invention provides a method of expressing a nucleotide sequence encoding a Cyt P450 or a functional derivative, homologue or equivalent thereof in a plant or cells of a plant, said method comprising introducing into said plant or cells of said plant a genetic construct in single or multicistronic form wherein at least one cistron encodes a Cyt b_5 or a mutant part, fragment or portion thereof or a functional and/or structural equivalent of homologue thereof; the genetic construct optionally further comprising cistrons encoding one or both of a Cyt P450 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent of homologue thereof; and/or a reductase or other associated protein.

More particularly, the present invention contemplates a method of expressing a genetic sequence encoding an F3'5'H or functional derivative, homologue or equivalent thereof in a plant or cells of a plant, said method comprising introducing a *difF* gene or enhancing expression of a *difF* gene or a functional derivative, homologue or equivalent thereof for a time and under conditions sufficient for the product of *difF* to enhance or otherwise modulate expression of a gene encoding F3'5'H.

The present invention further extends to introducing genetic constructs containing separately or together a *difF* and/or a gene encoding F3'5'H or derivatives, homologues or equivalents thereof.

Preferably, the modulation of *difF* expression is substantially exclusively in the flowers of plants.

Still another aspect of the present invention provides for the use of a genetic construct comprising a nucleotide sequence encoding a Cyt b_5 or a mutant part, fragment or portion thereof or a functional and/or structural equivalent or homologue thereof in the manufacture of a plant or cells of a plant in which said Cyt b_5 or a mutant part, fragment

or portion thereof or a functional and/or structural equivalent or homologue thereof enhances, modulates or otherwise facilitates expression of genetic material encoding a Cyt P450 or activity of a Cyt P450.

Still another aspect of the present invention is directed to the use of *difF* or a functional derivative, homologue or equivalent thereof in the manufacture of a genetic construct capable of enhancing, modulating or otherwise facilitating F3'5'H gene expression or F3'5'H activity.

The present invention also extends to flowers and cut flowers from transgenic plants and which comprises modified F3'5'H expression levels due to manipulation of *difF* expression. In particular, the present invention is directed to the modulation of flower colour. For example, various shades of blue flowers such as blue roses, carnations and chrysanthemums are contemplated by the present invention.

Although the present invention is particularly directed to the use of *difF* to enhance expression of F3'5'H, the manipulation of certain flower colours may require that the F3'5'H be down regulated. This can be effected by down regulating the expression of *difF* such as by antisense, co-suppression or ribozymes. Alternatively, an endogenous F3'5'H may be down regulated by targeting an endogenous *difF* and then an exogenous F3'5'H with an altered substrate specificity or activity introduced to again alter the flow of the metabolites to the flavonoid pathway. All such manipulations and modifications to the methods described therein are contemplated by the present invention.

The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figures 1a and 1b are schematic representations of the flavonoid biosynthesis pathways in *P. hybrida* flowers showing the enzymes and genetic loci involved in the conversions.

Enzymes involved in the pathway have been indicated as follows: PAL = phenylalanine ammonia-lyase; C4H = cinnamate 4-hydroxylase; 4CL = 4-coumarate: CoA ligase; CHS= chalcone synthase; CHI= chalcone isomerase; F3H= flavanone 3-hydroxylase; F3'H= flavonoid 3'-hydroxylase; F3'5'H= flavonoid 3'5' hydroxylase; FLS= flavonol synthase; DFR= dihydroflavonol-4-reductase; ANS= anthocyanin synthase; 3GT= UDP-glucose: anthocyanin-3-glucoside; 3RT= UDP-rhamnose: anthocyanidin-3-glucoside rhamnosyltransferase; ACT= anthocyanidin-3-rutinoside acyltransferase; 5GT= UDP-glucose: anthocyanin 5- glucosyltransferase; 3' OMT= anthocyanin *O*-methyltransferase; 3', 5' OMT=anthocyanin 3', 5' *O*-methyltransferase. Three flavonoids in the pathway are indicated as: P-3-G= pelargonidin-3-glucoside; DHM=dihydomyricetin; DHQ=dihydroquercetin. The flavonol, myricetin, is only produced at low levels and the anthocyanin, pelargonidin, is rarely produced in *P. hybrida.*

Figure 2 is a representation showing molecular analysis of *difF*. (A) Diagram showing the structure of *difF*. Exons are depicted as thick bars. The triangles indicate the position of *dTph1* insertions in the alleles *difF-V2082* and *difF-W2090.* (B) Phylogenetic tree showing the homology of the DIF-F protein to a variety of Cyt b_5 proteins. (C) Alignment of the DIF-F protein with Cyt b_5 from mammals, plants and yeast. Amino acids conserved in more than half of the sequences are indicated by black shading.

Figure 3 is a photographic representation showing Northern blot analysis of *difF* expression. (A) *difF* expression in different tissues and (B) in the corolla limb at different stages (1-6) of development (2) (C) *difF* expression in the corolla limb of wild type (R27) and mutant lines (W162, W115, W134) for the regulators *an1, an2* and *an11.* (D) *difF* mRNA corolla limbs homozygous for the mutable alleles *difF-V2082* or *difF-W2090* (m/m) and wildtype (+/+) siblings.

Figure 4 is a representation showing analysis of *difF* mutant flowers. (A) Phenotype of the *difF-W2090* allele in a $hf1^+rt^+$ (top) and a $hf1^+rt^-$ background (bottom). (B) PCR analysis of the *difF* locus in homozygous mutable (m/m) and revertant (+/m) sectors in flowers in different *hf1, hf2* and *rt* genotypes. The intermediately sized fragments are

heteroduplexes that consist of *difF::dTph1* and a *difF*$^+$ strand. (C) HPLC analysis of anthocyanin aglycones accumulated in the same sectors. The identity and the molar ratios of the anthocyanin peaks were established by chromatography of pure compounds: del, delphinidin; cya, cyanidin: peo, peonidin; mal, malvidin. (D) F3′5′H enzyme activity in the petal limbs of plants with the indicated phenotype selected from the backcross populations.

Figure 5 is a representation of the nucleotide sequence <400>1 and corresponding amino acid sequence <400>2 of *difF*. The triangle marks the position of an intron. The underlined sequences mark the insertion sites in the two mutant alleles.

Figure 6a is a diagrammatic representation of the binary plasmid pCGP1280, construction of which is described in Example xx.. Abbreviations are as follows: Tet = the tetracycline resistance gene; LB = left border; RB = right border; <u>sur</u>B = the coding region and terminator sequence from the acetolactate synthase gene; 35S = the promoter region from the cauliflower mosaic virus 35S transcript. Restriction enzyme sites are also marked.

Figure 6b to 6g are diagrammatic representations of intermediate plasmids used in the construction of pCGP1280 (Figure 6a). Restriction enzyme sites are also marked. Amp = ampicillin resistance gene.

Figure 6h is a schematic representation of the construction of the binary vector pCGP1280 (Figure 6a). pBS=pBluescript (Stratagene, USA). pANS= antocyanidin synthase promoter, anthocyanidin synthase terminator, Hfl=petunia flavonoid 3′5′ hydroxylase cDNA clone, 35S=cauliflower mosaic virus 35S promoter.

Figure 7a is a diagrammatic representation of the binary plasmid pCGP2355, construction of which is described in Example xx.. Abbreviations are as follows: Tet = the tetracycline resistance gene; LB = left border; RB = right border; <u>sur</u>B = the coding region and terminator sequence from the acetolactate synthase gene; 35S = the promoter region from the cauliflower mosaic virus 35S transcript. Restriction enzyme sites are also

marked.

Figures 7b to 7d are diagrammatic representations of intermediate plasmids used in the construction of pCGP2355 (Figure 7a). Restriction enzyme sites are also marked. Amp = ampicillin resistance gene.

Figure 7e A schematic representation of the construction of the binary vector pCGP2355 (Figure 7a). AntCHS=Antirrhinum (snapdragon) chalcone synthase promoter difF/DifF=petunia cytochrome b_5 cDNA clone, D8=petunia lipid transfer protein terminator.

Figure 8 is a photograph of transgenic and non-transgenic Exquisite carnation flowers. Transgenic Exquisite carnations transformed with the T-DNA contained in pCGP 1280 (b and c) produce flower of a similar colour to the non-transformed controls (a and d). Transgenic Equisite carnations transformed with the T-DNA contained in pCGP2355 (e and f) produce flowers of a novel colour in the violet to deep purple range. Colour photographs are available upon request to the Applicant.

Figure 9 is a representation of an autoradiograph of an RNA blot probed with ^{32}P-labelled fragments of the *Hfl* cDNA clone contained in pCGP602 (Holton *et al.*, 1993) and difF contained in pCGP2353. A ~1.8 kb Hfl transcript was detected in the petals of the transgenic Exquisite carnations transformed with the T-DNA contained in pCGP2355 (Lanes 2 to 5) or pCGP1280 (Lanes 6 to 9). The same size transcript ws detected in the positive controls of petunia petals of cultivars V30 (Lne 10) and Old Glory Blue (Lane 11). As expected no Hf1 transcript was detected in non-transgenic Exquisite petals (negative control) (Lane 1). A ~0.6 kb difF transcript was only detected in the petals of the transgenic Ezquisite carnations transformed with the T-DNA contained in pCGP2355 (Lanes 2 to 5) and in the positive controls of petunia petals, cultivar V30 and OGB (Lane 10 and 11), respectively. Each lane contained a ~10μg sample of total RNA. A photgraph of the ethidium bromide stained 255 rRNA band is shown as an indication of relative RNA levels.

EXAMPLE 1
GENETIC PROCEDURES

Northern blot, PCR and sequence analyses were done as previously described (2). The petunia lines W138 (relevant genotype: *an1-W138, hf1⁻, hf2⁻, rt⁻)* and V30 (relevant genotype: *An1⁺, Hf1⁺, Hf2⁺, Rt⁺)* were maintained as inbred stocks for several generations and were grown under standard greenhouse conditions. Transposon insertion alleles of *difF* were isolated in the W138 background as previously described (3) using primers complementary to *difF* and *dTphI* and maintained by selfing. In the backcrosses of the *difF* mutant lines with V30, segregation of the unstable *anI-W138* and the linked *rt⁻* allele were scored visually, while the anthocyanin substitution pattern was assayed by TLC and in a few selected plants by HPLC. Segregation of *hf1* and *hf2* alleles was determined by RFLP analysis (4) and by PCR amplification of the region containing the *dTphI* insertions for the mutant *difF* alleles.

^{32}P-Labelling of DNA Probes

DNA fragments (50 to 100 ng) were radioactively labelled with 50 μCi of [a-^{32}P]-dCTP using an oligolabelling kit (Bresatec). Unincorporated [a-^{32}P]-dCTP was removed by chromatography on a Sephadex G-50 (Fine) column.

DNA Sequence Analysis

DNA sequencing was performed using the PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kits from Applied Biosystems. The protocols supplied by the manufacturer were followed. The cycle sequencing reactions were performed using a Perkin Elmer PCR machine (GeneAmp PCR System9600) and run on an automated 373A DNA sequencer (Applied Biosystems).

Homology searches against Genbank, SWISS-PROT and EMBL databases were performed using the FASTA and TFASTA programs (Pearson and Lipman, 1988) or BLAST programs (Altschul *et al.*, 1990). Percentage sequence similarities were obtained using the LFASTA program (Pearson and Lipman, 1988). In all cases ktup values of 6 for nucleotide sequence

comparisons and 2 for amino acid sequence comparisons were used, unless otherwise specified.

Multiple sequence alignments (ktup value of 2) were performed using the ClustalW program incorporated into the MacVector™ 6.0 application (Oxford Molecular Ltd.).

Low stringency hybridization conditions

Hybridization conditions included a prehybridization step in 10% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulphate, 1% (w/v) SDS at 42°C for at least 1 hour. The ^{32}P-labelled fragments (each at $1x10^6$ cpm/mL) were then added to the hybridization solution and hybridization was continued at 42°C for a further 16 hours. The filters were then washed in 2 x SSC, 1% (w/v) SDS at 42°C for 2 x 1 hour and exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

RNA blots

Total RNA was isolated from the petal tissue of Exquisite carnation flowers using an RNAeasy kit from QIAGEN and following the protocols supplied by the manufacturer.

RNA samples were electrophoresed through 2.2 M formaldehyde/1.2% (w/v) agarose gels using running buffer containing 40 mM morpholinopropanesulphonic acid (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA (pH 8.0). The RNA was transferred to Hybond-N filters (Amersham) as described by the manufacturer.

Hybridization and washing conditions

RNA blots were probed with ^{32}P-labelled cDNA fragment (1×10^6 cpm/mL). Prehybridizations (1 hour at 42°C) and hybridizations (16 hours at 42°C) were carried out in 50% (v/v) formamide, 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate. Filters were washed in 2 x SSC, 1% (w/v) SDS at 65°C for 1 to 2 hours and then 0.2 x SSC, 1% (w/v) SDS at 65°C for 0.5 to 1 hour. Filters were exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

EXAMPLE 2
CHEMICAL PROCEDURES

Total anthocyanins of flower corolla sectors were extracted and hydrolysed by boiling in 1 ml 2N HCl for 30 min. The anthocyanin aglycones were analysed on a gradient HPLC system equipped with a Vydac C_{18} reversed phase column (5 μm; 250 x 4.6 mm) and a SPD-M10Avp diode array UV-detector (Shimadzu, Kyoto, Japan). Samples were eluted at 40°C, at a flow rate of 1 ml/min. Anthocyanins were monitored at 547 nm and dihydroflavonols at 280 nm. Solvent system used: a linear gradient elution for 22.5 min from 10 to 75% solvent B (1.5% phosphoric acid, 20% acetic acid and 25% acetonitrile in water) in solvent A (1.5% phosphoric acid in water). Anthocyanins were identified and quantified by comparison with the retention times and peak areas from standards. F3'5'H activity was measured with dihydroquercetin as a substrate as previously described (5), except that formation of the dihydromyricetin product was monitored by HPLC.

EXAMPLE 3
TRANSFORMATION PROCEDURES

A. tumefaciens transformations

The plasmids pCGP1280 or pCGP2355 (Figures 6a and 7a) are introduced into the *Agrobacterium tumefaciens* strain AGL0 by adding 5 μg of plasmid DNA to 100 μL of competent AGL0 cells prepared by inoculating a 50 mL MG/L (II) culture and growing for 16 hours with shaking at 28°C. The cells were then pelleted and resuspended in 0.5 mL of 85% v/v 100 mM $CaCl_2$/15% v/v glycerol. The DNA-*Agrobacterium* mixture was frozen by incubation in liquid N_2 for 2 minutes and then allowed to thaw by incubation at 37°C for 5 minutes. The DNA/bacterial mix was then placed on ice for a further 10 minutes. The cells are then mixed with 1 mL of LB (Sambrook *et al.*, 1989) media and incubated with shaking for 16 hours at 28°C. Cells of *A. tumefaciens* carrying pCGP1280 or pCGP2355 are selected on LB agar plates

containing 50 μg/mL tetracycline. The presence of pCGP1280 or pCGP2355 is confirmed by Southern analysis of DNA isolated from the tetracycline-resistant transformants.

Petunia transformations

(a) Plant Material

Leaf tissue from mature plants is treated in 1.25% w/v sodium hypochlorite for 2 minutes and then rinsed three times in sterile water. The leaf tissue is then cut into 25 mm^2 squares and precultured on MS media (13) supplemented with 0.05 mg/l kinetin and 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) for 24 hours.

(b) Co-cultivation of *Agrobacterium* Tissue

Agrobacterium tumefaciens strain AGL0 containing genetic material is maintained at 4°C on MG/L agar plates with 100 mg/L gentamycin. A single colony is grown overnight in liquid medium containing 1% w/v Bacto-peptone, 0.5% w/v Bacto-yeast extract and 1% w/v NaCl. A final concentration of 5 x 10^8 cells/ml is prepared the next day by dilution in liquid MS medium containing B5 vitamins (14) and 3% w/v sucrose (BPM). The leaf discs were dipped for 2 minutes into BPM containing AGL0/genetic material. The leaf discs are then blotted dry and placed on co-cultivation media for 4 days. The co-cultivation medium consists of SH medium (15) supplemented with 0.05 mg/l kinetin and 1.0 mg/l 2,4-D and included a feeder layer of tobacco cell suspension spread over the co-cultivation medium with a filter paper placed on top of the tobacco cell suspension.

(c) Recovery of transgenic plants

After co-cultivation, the leaf discs are transferred to a selection medium (MS medium supplemented with 3% w/v sucrose, α-benzylaminopurine (BAP) 2 mg/l, 0.5 mg/l α-naphthalene acetic acid (NAA), kanamycin 300 mg/l, 350 mg/L cefotaxime and 0.3% w/v Gelrite Gellan Gum (Schweizerhall)). Regenerating explants are transferred to fresh selection medium after 4 weeks. Adventitious shoots which survive the kanamycin selection are isolated and transferred to BPM containing 100

mg/l kanamycin and 200 mg/l cefotaxime for root induction. All cultures are maintained under a 16 hour photoperiod (60 μmol. m^{-2}, s^{-1} cool white fluorescent light) at 23± 2°C. When roots reach 2-3 cm in length the transgenic petunia plantlets are transferred to autoclaved Debco 51410/2 potting mix in 8 cm tubes. After 4 weeks, plants are replanted into 15 cm pots, using the same potting mix, and maintained at 23°C under a 14 hour photoperiod (300 μmol. m^{-2}, s^{-1} mercury halide light).

Flower Colour coding

The colour codes are taken from the Royal Horticultural Society's Colour Chart (RHSCC).

EXAMPLE 4

TRANSFORMATION OF *DIANTHUS CARYOPHYLLUS* CV. EXQUISITE

The binary vectors pCGP1280 or pCGP2355 were introduced into *A. tumefaciens* strain AGL0 cells, as described in Example . The pCGP1280/AGL0 or pCGP2355/AGL0 cells were used to transform carnation plants.

(a) Plant Material

Dianthus caryophyllus (cv. Exquisite) cuttings are obtained Baguely F & I, Flower and Plant Growers, Heatherton Road, Clayton South, Victoria, Australia. The outer leaves are removed and the cuttings are sterilised briefly in 70% v/v ethanol followed by 1.25% w/v sodium hypochlorite (with Tween 20) for 6 min and rinsed three times with sterile water. All the visible leaves and axillary buds are removed under the dissecting microscope before co-cultivation.

(b) Co-cultivation of *Agrobacterium* and *Dianthus* Tissue

Agrobacterium tumefaciens strain AGL0 (19), containing the binary vector pCGP1280 or pCGP2355, is maintained at 4°C on LB agar plates with 50 mg/L tetracycline. A single colony is grown overnight in liquid LB broth containing 50 mg/L tetracycline and

diluted to 5 x 10^8 cells/mL next day before inoculation. *Dianthus* stem tissue is co-cultivated with *Agrobacterium* for 5 days on MS medium supplemented with 3% w/v sucrose, 0.5 mg/L BAP, 0.5 mg/L 2,4-dichlorophenoxy-acetic acid (2,4-D), 100 mM acetosyringone and 0.25% w/v Gelrite (pH 5.7).

(c) Recovery of Transgenic *Dianthus* Plants

For selection of transformed stem tissue, the top 6-8 mm of each co-cultivated stem is cut into 3-4 mm segments, which were then transferred to MS medium (13) supplemented with 0.3% w/v sucrose, 0.5 mg/L BAP, 0.5 mg/L 2,4-D, 1 μg/L chlorsulfuron, 500 mg/L ticarcillin and 0.25% w/v Gelrite. After 2 weeks, explants are transferred to fresh MS medium containing 3% w/v sucrose, 0.16 mg/L thidiazuron (TDZ), 0.5 mg/L indole-3-butyric acid (IBA), 2 μg/L chlorsulfuron, 500 mg/L ticarcillin and 0.25% w/v Gelrite and care is taken at this stage to remove axillary shoots from stem explants. After 3 weeks, healthy adventitious shoots are transferred to hormone free MS medium containing 3% w/v sucrose, 5 μg/L chlorsulfuron, 500 mg/L ticarcillin, 0.25% w/v Gelrite. Shoots which survive 5 μg/L chlorsulfuron are transferred to the same medium for shoot elongation.

Elongated shoots are transferred to hormone-free MS medium containing 5 μg/L chlorsulfuron, 500 mg/L ticarcillin and 0.4% w/v Gelrite, in glass jars, for normalisation and root production. All cultures are maintained under a 16 hour photoperiod (120 mE/m^2/s cool white fluorescent light) at 23± 2°C. Normalised plantlets, approximately 1.5-2 cm tall, were transferred to soil (75% perlite/25% peat) for acclimation at 23°C under a 14 hour photoperiod (200 mE/m^2/s mercury halide light) for 3-4 weeks. Plants were fertilised with a carnation mix containing 1g/L $CaNO_3$ and 0.75 g/L of a mixture of microelements plus N:P:K in the ratio 4.7:3.5: 29.2.

EXAMPLE 5

TRANSFORMATION OF *ROSA HYBRIDA*

1. ***Rosa hybrida***

Plant tissues of the rose are transformed according to the method disclosed in PCT/AU91/04412, having publication number WO92/00371.

2. ***Rosa hybrida***

a. **Plant Material**

Kardinal shoots are used. Leaves are removed and the remaining shoots (5-6 cm) are sterilized in 1.25 % w/v sodium hypochlorite (with Tween 20) for 5 minutes followed by three rinses with sterile water. Isolated shoot tips are soaked in sterile water for 1 hour and precultured for 2 days on MS medium containing 3% w/v sucrose, 0.1 mg/L BAP, 0.1 mg/l kinetin, 0.2 mg/l Gibberellic acid, 0.5% w/v polyvinyl pyrrolidone and 0.25% w/v Gelrite Gellan Gum, before co-cultivation.

b. **Co-cultivation of *Agrobacterium* and *Rosa* shoot Tissue**

Agrobacterium tumefaciens strains ICMP 8317 (18) and AGL0, containing genetic a particular construct are maintained at 4°C on MG/L agar plates with 100 mg/L gentamycin. A single colony from each *Agrobacterium* strain is grown overnight in liquid MG/L broth. A final concentration of 5 x 10^8 cells/ml is prepared the next day by dilution in liquid MG/L. Before inoculation, the two *Agrobacterium* cultures are mixed in a ratio of 10:1. A longitudinal cut is made through the shoot tip and an aliquot of 2 μl of the mixed *Agrobacterium* cultures is placed as a drop on the shoot tip. The shoot tips are co-cultivated for 5 days on the same medium used for preculture.

Agrobacterium tumefaciens strain AGL0 is maintained at 4°C on MG/L agar plates with 100 mg/L kanamycin. A single colony from each *Agrobacterium* strain is grown overnight in liquid MG/L broth. A final concentration of 5 x 10^8 cells/ml is prepared the next day by dilution in liquid MG/L.

c. Recovery of Transgenic *Rosa* Plants

After co-cultivation, the shoot tips are transferred to selection medium. Shoot tips are transferred to fresh selection medium every 3-4 weeks. Galls observed on the shoot tips are excised when they reached 6-8 mm in diameter. Isolated galls are transferred to MS medium containing 3% w/v sucrose, 25 mg/l kanamycin, 250 mg/l cefotaxime and 0.25% w/v Gelrite Gellan Gum for shoot formation. Shoots regenerated from gall tissue are isolated and transferred to selection medium. GUS histochemical assay and callus assay are used to identify transgenic shoots. Transgenic shoots are transferred to MS medium containing 3% w/v sucrose, 200 mg/l cefotaxime and 0.25% w/v Gelrite Gellan Gum for root induction. All cultures are maintained under 16 hour photoperiod (60 μE cool white fluorescent light) at 23$\pm$ 2°C. When the root system is well developed and the shoot reached 5-7 cm in length the transgenic rose plants are transferred to autoclaved Debco 514110/2 potting mix in 8 cm tubes. After 2-3 weeks plants are replanted into 15 cm pots using the same potting mix and maintained at 23°C under a 14 hour photoperiod (300 μE mercury halide light). After 1-2 weeks potted plants are moved to glasshouse (Day/Night temperature : 25-28°C/14°C) and grown to flowering.

EXAMPLE 6

TRANSFORMATION OF *CHRYSANTHEMUM MORIFOLIUM*

a. Plant Material

Chrysanthemum morifolium cuttings are obtained. Leaves are removed from the cuttings, which were then sterilized briefly in 70% v/v ethanol followed by 1.25% w/v sodium hypochlorite (with Tween 20) for 3 minutes and rinsed three times with sterile water. Internodal stem sections are used for co-cultivation.

b. Co-cultivation of *Agrobacterium* and *Chrysanthemum* Tissue

Agrobacterium tumefaciens strain LBA4404 (Hoekema *et al* 1983), containing is grown on MG/l agar plates containing 50 mg/l rifampicin and 10 mg/l gentamycin. A single colony from each *Agrobacterium* is grown overnight in the same liquid

medium. These liquid cultures are made 10% v/v with glycerol and 1 ml aliquots transferred to the freezer (-80°C). A 100-200μl aliquot of each frozen *Agrobacterium* is grown overnight in liquid MG/l containing 50 mg/l rifampicin and 10 mg/l gentamycin. A final concentration of 5 x 10^8 cells/ml is prepared the next day by dilution in liquid MS containing 3% w/v sucrose. Stem sections are co-cultivated with *Agrobacterium* in co-cultivation medium for 4 days.

c. Recovery of Transgenic *Chrysanthemum* Plants

After co-cultivation, the stem sections were transferred to selection medium. After 3-4 weeks, regenerating explants are transferred to fresh medium. Adventitious shoots which survive the kanamycin selection are isolated and transferred to MS medium containing kanamycin and cefotaxime for shoot elongation and root induction. All cultures are maintained under a 16 hour photoperiod (80 μE cool white fluorescent light) at 23 ± 2°C. Leaf samples are collected from plants which rooted on kanamycin and Southern blot analysis is used to identify transgenic plants. When transgenic chrysanthemum plants reach 4-5 cm in length, they are transferred to autoclaved Debco 51410/2 potting mix in 8 cm tubes. After 2 weeks, plants are replanted into 15 cm pots using the same potting mix and maintained at 23°C under a 14 hour photoperiod (300 μE mercury halide light). After 2 weeks potted plants are moved to glasshouse (Day/Night temperature : 25-28°C/14°C) and grown to flowering.

EXAMPLE 7
PLANTS

The Exquisite carnation was employed for transformation experiments (Baguley F&I, Flower & Plant Growers, Heatherton Road, Clayton South, Victoria, Australia). this carnation has a bicoloured flower.

EXAMPLE 8
IDENTIFICATION OF *difF*

To identify additional genes involved in anthocyanin modification, the inventors isolated cDNAs corresponding to genes that are down-regulated in flowers with a mutation in the regulatory *anthocyanin-1 (an1)* gene (6). Based on its sequence, one gene, potentially involved in flavonoid hydroxylation, was chosen for detailed analysis. This gene, termed herein *"difF"*, encodes a polypeptide of 149 amino acids that represents a novel class of plant Cyt b_5 proteins (Fig. 2b, c). The highest degree of similarity is clustered around the pairs of histidine residues (His-39 and His-63) that correspond to the axial ligands for heme binding (7). Although the Cyt b_5 sequences show strong divergence in the C-terminal part of the polypeptide, they have a strikingly similar hydropathy plot. This hydrophobic C-terminal part anchors the enzyme to the endoplasmic reticulum (ER) membrane (7).

EXAMPLE 9
EXPRESSION OF *difF*

To examine the function of *difF* in anthocyanin biosynthesis, its expression pattern was analysed by Northern blots and compared to the expression pattern of the *dfr* gene, encoding dihydroflavonol 4-reductase, a key enzyme of the anthocyanin pathway. Fig. 3A shows that the *difF* transcripts accumulate in the limb and tube of the flower corolla and in the ovaries, but not in vegetative organs such as leaves, root and stem. During petal development the temporal *difF* expression pattern closely matches that of the gene encoding dihydroflavonal reductase *(dfr)*, with both transcripts reaching a maximum around stage 3, when the flower bud starts to open (Fig. 3B). To test if *difF* expression is controlled by any of the known regulators of the anthocyanin pathway, the inventors analysed *difF* transcript levels in stage 3 flowers (2) of the corresponding mutants. Fig. 3C shows that *difF* expression is down-regulated in petal limbs of *an1*$^-$, *an2*$^-$ and *an11* mutants, when compared to wildtype. Although *an2-W115* is a null allele, this mutation reduces anthocyanin

synthesis strongly, but does not block it completely. This indicates that *an2* function is partially redundant and explains the residual *difF* and *dfr* transcripts detected in *an2-W115* petal limbs (Fig. 3C). Taken together, these data show that the spatio-temporal and genetic control of *difF* expression are consistent with a role in athocyanin synthesis.

EXAMPLE 10
ISOLATION OF *difF* MUTANTS

To establish the *in vivo* function of *difF,* the inventors isolated *difF* mutants by a PCR based screen (3) to identify plants of the line W138 in which a *dTph1* transposon had inserted in the *difF* gene. Among 4000 W138 plants, the inventors found that two individuals that were heterozygous for the wildtype *difF*$^{+}$ allele and a transposon insertion derivative (*difF-V2082* and *difF-W2090* respectively). Seeds were germinated from these individuals, that had been produced by self-pollination, and progeny homozygous for *difF-V2082* and *difF-W2090* identified by PCR. Sequence analysis showed that in *difF-V2082* a 284 bp *dTph1* element had inserted in the first exon, 10 bp upstream of the splice-site, thereby disrupting the protein coding sequence. The *difF-W2090* allele contained a 284 bp *dTph1* insertion in the middle of exon 2, that also disrupts the coding sequence, see Figure 5 for mapping of insertions. Northern analysis showed that flowers of *difF-V2082* homozygous progeny accumulated *difF* transcripts that are about 300 bp larger than the wildtype *difF* transcript (Fig. 3D). By analogy to other *dTph1* insertion alleles this mutant transcript is likely to contain the transcribed *dTph1* sequence. In *difF-W2090* homozygotes the amount of *difF* mRNA was reduced about three-fold when compared to *difF*$^{+}$ siblings. Since *difF-W2090* is relatively unstable, these transcripts most likely result from *dTph1* excisions and probably contain different transposon footprints.

EXAMPLE 11

MUTATIONS OF *difF* AFFECTS FLOWER COLOUR

To study the effects of mutant *difF* alleles into an $hf1^+$ or $hf2^+$ genetic background, the inventors made backcrosses with line V30 ($hf1^+$, $hf2^+$, $an1^+$, rt^+), using *difF* mutant lines as the recurrent parent. As expected, these progenies (co-)segregated 1:1 for $an1^{mutable}$ mutable ($an1^T$) and ^-rt plants (Table 1). If the 5′ substitution of anthocyanin is dependent on the segregation of *hf1* and *hf2* alone, one would expect to find plants accumulating malvidin ($hf1^+$ $hf2^+$ and hf^+, $hf2^-$), malvidin plus peonidin ($hf1^-$, $hf2^+$; the relatively weak *hf2* locus enables the 5′ substitution of only about 50% of the anthocyanins) or peonidin ($hf1^-$ $hf2^-$) corolla pigments in a ratio 2:1:1. However, combined results of the two backcross populations segregating for *difF-V2082* and *difF-W2090*, respectively showed a segregation ratio of 38:51:6 (Table 1). This suggested that a third mutant gene segregated that reduced the 5′ substitution, possibly *difF*. To test this directly, the inventors subjected representative plants of the various phenotypic classes to Southern blot and PCR analyses to determine the *hf1*, *hf2* and *difF* genotype. This revealed that the malvidin accumulating plants were all $hf1^+$ $difF^+$, while those accumulating a mixture of malvidin and peonidin were either $hf1^+$, $difF^m$, $hf1^-$ $hf2^+$ $difF^m$ or $hf1^-$ $hf2^+ difF^+$.

Closer inspection showed that the $hf1^+$ rt^+ individuals which were homozygous for the *difF-W2090* allele had variegated flowers with purple (revertant) sectors and spots on a purplish magenta (mutant) background (Fig. 4A top). Also flowers of $hf1^-$ $hf2^+$ $difF^m$ siblings were variegated, although the colour difference between mutant and revertant tissue was less pronounced. In an $hf1^+$ rt^- plants the variegation was seen as "dull-grey" revertant spots and sectors on a "dull-red" mutant background (Fig. 4A bottom). To test whether these variegated flower colours were due to genetic instability of the *difF-W2090* allele, the inventors isolated DNA from several large revertant petal sectors and from the mutant corolla sectors and analysed the *difF* gene by PCR. Fig. 4B shows that reversion of the flower colour are associated with (somatic) excision of the *dTph1* element from *difF-W2090*. Also *difF-V2068*

individuals had variegated flowers, but the frequency of revertant spots was lower by at least one order of magnitude.

EXAMPLE 12

MUTATION OF *difF* REDUCES MODIFICATION OF THE ANTHOCYANIN

To examine how the *difF* mutation affected flower colour, the inventors dissected (isogenic) *difF*$^+$ revertant and *difF* mutant sectors of single flowers and analysed the anthocyanin aglycones by HPLC. Some representative chromatograms are shown in Fig. 4C. In *difF*$^+$ revertant petal sectors on *hf1* $^-$ *rt* plants about 80% of the anthocyanins are 3′, 5′ substituted (delphinidin), while in *dif*^{m} mutant sectors of the same flower this amount is reduced to about 40% (Fig. 4C, top). The reduced delphinidin accumulation is correlated with an increase in the accumulation of 3′ substituted and anthocyanin (cyanidin) from 20 to 63%. This indicates that the *difF* mutation reduced the formation of 3′, 5′ hydroxylated anthocyanins by about 50% and that the remaining precursors are converted into a 3′ hydroxylated anthocyanin. The same phenomenon was observed in *hf1*$^+$, *rt*$^+$ flowers. In this background, the *difF-W2090* mutation reduced the fraction of 3′, 5′ substituted anthocyanins (malvidin) from 94 to 73%, which correlated with an increase in 3′ substituted anthocyanin (peonidin) from 6 to 27% (Fig. 4C, middle). This indicated a 25% inhibition in the formation of 3′, 5′ substituted anthocyanins. In *hf1*$^-$ *hf2*$^+$ tissue less than half of the anthocyanins were 3′,5′ substituted (44%), possibly because the *hf2* locus expresses lower amounts of F3′5′H protein, or a F3′5′H protein with lower activity. In this background a *difF* mutation decreased 3′,5′ substitution further down to 29%, corresponding to about 35% inhibition (Fig. 4C, bottom).

EXAMPLE 13

difF MUTATION REDUCES F3′5′H ACTIVITY

To test if *difF* stimulates 3′, 5′ substitution of anthocyanin precursors by regulating

the activity of the CytP450 enzyme F3′5′H, the inventors measured F3′5′H activities in different genotypes. Because this required larger quantities of petal tissue, these measurements could not be performed on (isogenic) mutant and revertant sectors of single flowers. Instead, the inventors selected two or three plants from the V30 backcross populations for each genotype and determined F3′5′H enzyme activity in microsomes that were isolated from stage 4 petal limbs. Fig. 4D shows that the *difF* mutation reduced *hf1* encoded F3′5′H activity by about 20-fold, while *hf2* encoded F3′5′H activity was reduced approximately 3-fold.

The data show unequivocally that *in vivo,* a Cyt b_5 (DIF-F) is required for activity of Cyt P450, F3′5′H, without an apparent effect on other Cyt P450 enzymes, such as those involved in 3′-hydroxylation of dihydroflavonols, synthesis of the flavonoid precursor cinnamic acid, or synthesis of hormones controlling plant development. Both *in vitro* reconstruction experiments (7) as well as *in vivo* over-expression experiments in yeast (8) and human cells (9) have shown that the activity of a Cyt P450 can be increased 10 to 20 fold by co-expression of a Cyt b_5. Therefore, *difF* may provide a critical tool to increase the activity of a *f3′5′h* transgene in ornamental flowers that normally lack blue colours.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

EXAMPLE 14

SCREENING FOR EFFECTS OF CYT B_5 ON P450 MOLECULES

The effects of Cyt b_5 molecules on different P450 molecules such as F3′5′H or F3′H is determined using yeast. Details of suitable yeast strains and expression vectors is sshown in US Patent No. 5,349,125. In one embodiment, the petunia Cyt b_5 is incorporated into the genome of a yeast and genetic constructs encoding a Cyt P450 introduced into the cell. Expression of Cyt P450 may be measured by any number of ways. In relation to F3′5′H, for example, radiolabelled dihydrokoenpferol (DHK) or radiolabelled naringinin may be used. For other P450's, the product or substrate may be measured using, for example, HPLC, TLC or other suitable procedures.

Exquisite is a carnation cultivar that produces bi-coloured flowers with a deep red centre and a pale pink rim. The petals normally accumulate cyanidin, a 3′,4′-hydroxylated anthocyanidin and the flavonolos quercetin and kaempferol (a 3′,4′-hydroxylated flavonol and 4′-hydroxylated flavonol, respectively).

Introduction of a petunia flavonoid 3′5′ hydroxylase (F3′5′H) under the control of a carnation ANS (anthocyanidin synthase) promoter (contained in pCGP1280 [Figure 6a]) results in either no or a slight alteration of petal colour with low levels of delphinidin (3′,4′,5′-hydroxylated anthocyanidin) [Figure 1a and 1b] (Tables 2 and 3) being produced.

Introduction of the same chimeric F3′5′H (ANS:HF1:ANS) along with difF under the control of a snapdragon CHS (chalcone synthase) promoter both contained in pCGP2355 (Figure 7a) resulted in a major shift in flower colour. The flowers of the transgenic Exquisite/pCGP2355 flowers were deep purple with a pale purple rim (figure 8). HPLC analysis of the anthocyanidins and flavonol content of Exquisite/pCGP2355 showed that delphinidin (the 3′,4′,5′-hydroxylated anthocyanidin) was the predominant anthocyanidin produced.

This result suggested that expression of the introduced difF along with the F3′5′H chimeric gene enhanced F3′5′H activity so that higher levels of delphinidin were produced compared to the expression of the F3′5′H chimeric gene with the absence of $cytb_5$.

TABLE 1

SUMMARY W138(difF) X V30 CROSSES

Table 1: Number of plants with flower colour and anthocyanins in the backcrosses (W138::difF^m x V30)x W138::difF^m

	white with coloured spots (anl^m)						full coloured (Anl$^\pm$)					
	Rt$^+$			rt$^-$			Rt$^+$			rt$^-$		
cross*	mal	mal/peo	peo	del	del/cya	cya	mal	mal/peo	peo	del	del/cya	cya
Z2363	44						49					
	4			39			48			1		
	3	0	0	15	14	7	16	22	3	0	1	0
Z2364	79						61					
	3			73			59			2		
	1	1	1	19	12	17	12	29	3	0	1	0
total	121						110					
	7			112			107			3		
	4	1	1	34	36	24	38	51	6	0	2	0

*backcross family Z2363 segregated for *difF-V2082*, and Z2364 segregated for *difF-W2090*

Table 2

Cultivar	pCGP	Construct	Acc#	RHSCC	Del	Cya	Del	M	Q	K	M
					mg/g	mg/g	%	mg/g	mg/g	mg/g	%
Exquisite	inner	control	20173 (b)	56a	0.00	1.23	0.0%	0.00	0.89	1.09	0.0%
Exquisite	rim	control	20173 (b)		0.00	1.03	0.0%	0.00	1.28	2.20	0.0%
Exquisite	whole	control	20173 (b)	56a	0.00	0.62	0.0%	0.00	0.65	0.95	0.0%
Exquisite	1280 rim	Ans-Hf1-Ans	20183		0.25	1.13	18.4%	0.13	1.22	2.16	3.8%
Exquisite	2355 rim	Ans-Hf1-Ans/AntCHS-cytb5-D8	19787		0.38	0.27	58.2%	0.09	0.72	1.45	4.1%
Exquisite	2355 rim	Ans-Hf1-Ans/AntCHS-cytb5-D8	19788		0.02	1.69	0.9%	0.00	0.92	2.25	0.0%
Exquisite	2355 rim	Ans-Hf1-Ans/AntCHS-cytb5-D8	19789		0.65	0.08	88.8%	0.14	0.89	1.50	5.9%
Exquisite	2355 rim	Ans-Hf1-Ans/AntCHS-cytb5-D8	19794		0.45	0.18	71.1%	0.11	0.76	1.59	4.7%
Exquisite	2355 rim	Ans-Hf1-Ans/AntCHS-cytb5-D8	19796		0.38	0.19	67.4%	0.11	1.07	1.58	4.2%
Exquisite	2355 rim	Ans-Hf1-Ans/AntCHS-cytb5-D8	19809		0.64	0.08	89.0%	0.26	0.60	1.66	11.5%
Exquisite	2355 rim	Ans-Hf1-Ans/AntCHS-cytb5-D8	19812		0.63	0.36	63.8%	0.10	0.93	1.72	3.8%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19810	71a	2.47	0.30	89.3%	0.37	1.71	2.97	7.9%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19810	71a	1.04	0.26	80.2%	0.22	0.56	0.92	14.8%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19812	71a	0.66	0.61	52.1%	0.09	0.83	1.14	4.6%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19788	187c	0.01	1.39	0.7%	0.00	0.63	1.04	0.0%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19789	83a	0.81	0.34	70.3%	0.12	0.87	0.97	6.5%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19794	79a	0.54	0.35	60.4%	0.07	0.63	0.81	4.9%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19796	79a	0.47	0.40	54.0%	0.09	0.95	0.96	4.7%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19802	64a	0.43	0.63	40.9%	0.07	0.67	0.80	4.7%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19805	79a	0.42	0.36	53.9%	0.06	0.68	0.75	4.2%
Exquisite	2355 inner	Ans-Hf1-Ans/AntCHS-cytb5-D8	19806	71a	0.80	0.59	57.6%	0.09	0.86	1.06	4.7%

Table 3

Cultivar	pCGP	Construct	Acc#	RHSCC	Anthocyanidins			Flavonols			
					Del	Cya	Del	M	Q	K	M
					mg/g	mg/g	%	mg/g	mg/g	mg/g	%
Exquisite	1280	Ans-Hf1-Ans	20186	64b	0.01	1.10	0.8%	0.00	0.88	1.71	0.0%
Exquisite	1280	Ans-Hf1-Ans	20196	64a	0.01	1.36	0.7%	0.00	0.89	1.65	0.0%
Exquisite	1280	Ans-Hf1-Ans	20181	64a	0.01	1.40	0.7%	0.00	2.24	2.06	0.0%
Exquisite	1280	Ans-Hf1-Ans	20184	74a	0.04	0.87	4.1%	0.00	0.86	1.13	0.0%
Exquisite	1280	Ans-Hf1-Ans	20194	64a	0.02	1.23	1.3%	0.00	1.17	1.78	0.0%
Exquisite	1280	Ans-Hf1-Ans	20198	61a	0.42	1.27	24.8%	0.11	1.36	2.50	2.8%
Exquisite	1280	Ans-Hf1-Ans	20189	61a	0.16	1.75	8.5%	0.00	0.86	2.00	0.0%
Exquisite	1280	Ans-Hf1-Ans	20194	64a	0.03	1.60	1.7%	0.00	1.32	2.02	0.0%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19786	61a	0.01	1.28	0.6%	0.00	0.96	1.98	0.0%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19818	79a	0.81	0.12	86.9%	0.19	0.72	1.29	9.4%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19815	72a	0.71	0.42	63.0%	0.12	0.85	1.26	5.7%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19804	71a	0.56	0.27	67.4%	0.11	0.95	1.45	4.6%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19802	64a	0.73	0.66	52.5%	0.16	1.11	2.11	5.0%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19806	71a	0.97	0.56	63.5%	0.17	1.31	1.97	5.2%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19808	71a	1.83	0.62	74.7%	0.27	1.57	2.48	6.7%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19810	71a	1.73	0.19	89.9%	0.28	1.64	3.05	6.0%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19808	71a	1.26	0.41	75.6%	0.20	1.15	2.02	6.3%
Exquisite	2355	Ans-Hf1-Ans/AntCHS-cytb5-D8	19819	71a	0.64	0.25	71.9%	0.09	0.42	0.37	11.3%
Exquisite	Aglo	control	21129	56a	0.00	1.68	0.0%	0.00	2.52	2.38	0.0%
Exquisite	Aglo	control	21129	56a	0.00	1.40	0.0%	0.00	1.27	1.75	0.0%
Exquisite	Aglo	control	21129	56a	0.00	1.44	0.0%	0.00	1.48	1.86	0.0%

TABLE 4.....

ANSpromoter -> Restriction Map

```
DNA  sequence    2552 b.p.   GAATTCACTCGA ... TCATAATCTAGA   linear

                                                                                  Sau3A I
                                                                                  Mbo I
                                                                                  Dpn I
                                      Ple I                                      Bcl I
 EcoR I   Taq I                       Hinf I                   Mnl I            Mae II
 |        |                           |                        |                |  ||
5' GAATTCACTCGAAATGATACTGTGAGTCTGCGATAGGCTCGTTTTGAGGCGGAAATTATGATTTTACGACGTGATCAATT  80
   CTTAAGTGAGCTTTACTATGACACTCAGACGCTATCCGAGCAAAACTCCGCCTTTAATACTAAAATGCTGCACTAGTTAA
 |        |.           .          |        .         .         |   .         .  |. ||      .
 1        9                       24                           46               69
                                  24                                              72
                                                                                   73
                                                                                   73
                                                                                   73

                       Mbo II
         Mse I         Bbv II                                        HgiA I
   Nla III         Hga I   Dde I                                    Bsp1286 I
   |     |         | ||    |                                        |
 GAGCATGACTTAAATTTGCGTCTTCTCAGTCGTCGTTGCATTGCAATTTTTAGTATTTTCAGGTGCTCTGAAAGTGTTTA  160
 CTCGTACTGAATTTAAACGCAGAAGAGTCAGCAGCAACGTAACGTTAAAAATCATAAAAGTCCACGAGACTTTCACAAAT
   |     |         | ||    |      .        .        .        .    |      .        .
   84              98      105                                      143
         90          100                                            143
                      101

                                      Taq I
                                      Sal I
          Mse I                       HinC II   Mbo II                     Bsp1286 I
 Rsa I    Dra I      EcoR V           Acc I    Ear I       Mse I     Mae II
 |        ||         |                ||       ||          |         | |
 GTACATCGTTTTTAAAATGGATATCTTTTTGTTCTGGTCGACTTACTCTTCGCTTTTTAATGCAGACGTGCCCGTTATTG  240
 CATGTAGCAAAAATTTTACCTATAGAAAAACAAGACCAGCTGAATGAGAAGCGAAAAATTACGTCTGCACGGGCAATAAC
 |       .||         |        .       ||.      ||  .        | .      | |  .       .
 161      171        180              197      206         217       226
           172                        197       207                   228
                                      197
                                       198

  Mae II                                                                        Mae I
   Afl III                                                       Mnl I        Spe I
  BsaA I                             Hga I                      Taq I       Rsa I
  ||                                 |                          | |         | ||
 CTACGTGTATTCACAAAGGTATGACCGTGTTCTGTAGCGTCTAATGATAATATATGAAGTCGAGGTTGCATTTGTACTAG  320
 GATGCACATAAGTGTTTCCATACTGGCACAAGACATCGCAGATTACTATTATATACTTCAGCTCCAACGTAAACATGATC
  ||     .         .         .       |  .         .             | |       .  | ||  .
  242                                277                        300         314
   243                                                             302        316
   243                                                                         317

                                        Sau3A I  Hph I
                                        Mbo I   Mae III
                                        Dpn I  BstE II
                                      Mae I  Mnl I              Mbo II              Mnl I
                                      |  |   | |||              |                   |
 TCCGATAATAATTAGTATCGTTTTCATACTGATACTAGATCGGAGGTCACCATACCCGTGAAGATTTTTCTGTGAGAGGA  400
 AGGCTATTATTAATCATAGCAAAAGTATGACTATGATCTAGCCTCCAGTGGTATGGGCACTTCTAAAAAGACACTCTCCT
         .         .         .        |  | . | |||  .           |             .    |  .
                                      355   363                 380                 396
                                         358  365
                                         358   366
                                         358    367

                                          Hga I           Mse I
     Sty I                                Aha II          Hpa I
     Sec I    Mnl I            Nla III                    HinC II
     |        |                |       |                  ||
 AAAGAACCCAAGGACGAGGTTCAAATCTACACATGGAAAGACGCCACGCTTCGTGAGTTAACTGACCTTGTATGTCGCCA  480
 TTTCTTGGGTTCCTGCTCCAAGTTTAGATGTGTACCTTTCTGCGGTGCGAAGCACTCAATTGACTGGAACATACAGCGGT
     | .      |   .         . |       |         .         ||.          .         .
     408      416              432                        457
     408                               440                457
                                       440                 458
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ANSpromoter -> Restriction Map

```
           HinP I                                                Nla III
           Hha I                                                NspH I
  Dde I    Hae II                                               Nsp7524 I
  Esp I    Eco47 III          Hph I      Fok I     PflM I       Afl III
  ||       ||                 |          |         |            ||
TCGCTTAGCGTAGCGCTGAACATCGTTTTCACCCTGCTCCATCCATCAACCATTTATTGGTCTTATACATGTGTGATTGC   560
AGCGAATCGCATCGCGACTTGTAGCAAAAGTGGGACGAGGTAGGTAGTTGGTAAATAACCAGAATATGTACACACTAACG
  ||     • ||        •        |•         |         |         •      || •         •
  483      492                509        520       530              547
   484     492                                                      547
            493                                                     547
            493                                                      548

                                Fnu4H I
                                Bbv I
                               Alu I
                               Pvu II     Ple I  SfaN I
                            Gsu I     Mae I  Taq I                        Xca I
                Hph I   Mae II     NspB II    Hinf I                      Acc I
                |       |     |  |||     |   |  |   |                       |
GTTGTTCTTACATTTAGGTGAAAGACGTTTCTCCAGCTGCTAGGAGTCGAGATGCGAAATTGTCGTTTGCGACTGTATAC   640
CAACAAGAATGTAAATCCACTTTCTGCAAAGAGGTCGACGATCCTCAGCTCTACGCTTTAACAGCAAACGCTGACATATG
         •      | •     |    •|  |||     |   |  |  •|         •         •  |     •
                577     585      594         604                          635
                              591     600      607                        635
                                 594         604    611
                                  595
                                   596
                                   596

           Nla III
          Sph I
          NspH I
          Nsp7524 I
         Nsi I
        SfaN I
       Fok I                        Mbo II                 BsmA I            Tth111 I
       ||| ||                       |                      |                   |
CTTGATAGAAATGGATGCATGCAAGTAAAGAAGGTATCTTCTAATTCATCTTTCGTAGAGACATAGCGTGAATTTGGACG   720
GAACTATCTTTACCTACGTACGTTCATTTCTTCCATAGAAGATTAAGTAGAAAGCATCTCTGTATCGCACTTAAACCTGC
     •     ||| || •         •       | •         •          | •         •       | •
           653                      677                    698                 717
            654
             655
              657
              657
              657
               658

                             Mae II
                            SnaB I
                            BsaA I
                        Alu I                 Hph I
                        |    ||               |
GGGTCTTTGGTTTGAGAAAGATAACAGCTTTACGTATTTTTGTAGATGGGTGAAACCTTTTCAAATCCGTATAAGCGTAA   800
CCCAGAAACCAAACTCTTTCTATTGTCGAAATGCATAAAAACATCTACCCACTTTGGAAAAGTTTAGGCATATTCGCATT
         •         •    |   •||         •     |•         •         •         •
                        746                   769
                              751
                              751
                               752

                                                                     Sau3A I
                                                                     Mbo I
                                                                     Dpn I
        Bsr I                               SfaN I         BsmA I   Bcl I
        |                                   |              |        ||
AGACGACAACTGGGCTTTAGGGGACACATTCTTTCAGGTATAATTGATGCGACTAACAATAGTCTCCACTGATCATATTC   880
TCTGCTGTTGACCCGAAATCCCCTGTGTAAGAAAGTCCATATTAACTACGCTGATTGTTATCAGAGGTGACTAGTATAAG
        |•         •         •         •    |    •        • |       ||        •
        809                                 846             862     870
                                                                     871
                                                                     871
                                                                     871
```

ANSpromoter -> Restriction Map

```
  Mbo II    Taq I
 Ear I  Mae II                         Acc I                      Mae II
TACTCTTCTACGTTCGATACTGACTGTTTCTGGTTATTTGGTAGACAGGAGATTATTTGGACGTAGCAATTCAGTAGCGT 960
ATGAGAAGATGCAAGCTATGACTGACAAAGACCAATAAACCATCTGTCCTCTAATAAACCTGCATCGTTAAGTCATCGCA
883     890                            921                        941
 884        894
             Mae II
             Afl III
            Pml I                          Mae I
            BsaA I                        Sty I
           Afl III                        Sec I
                                          Avr II
AGAGATGTTTCCACACGTGTTATCGTAAAAGAAGCAAGATAAGCCTAATGCCTAGGGTGGTGGTATGACTTCCGTTGCTT 1040
TCTCTACAAAGGTGTGCACAATAGCATTTTCTTCGTTCTATTCGGATTACGGATCCCACCACCATACTGAAGGCAACGAA
           973                            1011
            974                           1011
            974                           1011
             975                           1012
             975
  Sau3A I
  Mbo I
  Dpn I
 Pvu I
 Taq I                                              Mbo II       Nla III
Cla I    Tth111 II                                Mse I    Mae I    BspH I
ATCGATCGTGCTTGTAAGTAATTTCCGTCTTATCTTTTCCTGTTATATAAAGTTAATCTTCTCTAGGACTTTCATGAACC 1120
TAGCTAGCACGAACATTCATTAAAGGCAGAATAGAAAAGGACAATATATTTCAATTAGAAGAGATCCTGAAAGTACTTGG
1041     1049                                     1093      1103     1112
 1042                                                 1097            1113
  1043
   1044
   1044
   1044
                                                        Mae I
              Sau3A I                                   Spe I
              Mbo I                                  Xca I
              Dpn I           Alu I                  Acc I
 Tth111 II   Taq I   Nla III   Mae I    Mse I   Mae II                     Mse I
TTGTTTGTGTATTTATTTCTCGATCAACATGATAGAGCTAGTTTTTAAGCAACGTATACTAGTAGTCTATTGGAAGTTAA 1200
AACAAACACATAAATAAAGAGCTAGTTGTACTATCTCGATCAAAAATTCGTTGCATATGATCATCAGATAACCTTCAATT
 1122          1140   1148     1158     1165   1172                         1197
                1142         1156                 1174
                1142                              1174
                1142                                  1178
                                                       1179
               Sau3A I
               Mbo I
               Dpn I         Nla III
               Alw I        NspH I
       Mse I   Rsa I        Nsp7524 I             Mae I     Ple I
                                               Mbo II      Hinf I
GACACGGTTCTTAAAAAGGTACGATCCAAGTGAAGCATGTTAGATATGACACTTTCTTCTAGGGACGACTCTCGTATGCC 1280
CTGTGCCAAGAATTTTTCCATGCTAGGTTCACTTCGTACAATCTATACTGTGAAAGAAGATCCCTGCTGAGAGCATACGG
       1211    1219         1235                 1255        1267
                1223        1235                    1259     1267
                1223         1236
                1223
                1223
                                                  Fok I
                                                 SfaN I
                                                Nsi I       BsmA I
ACCCGACTTTTTCAATTTTTTTTGTGAATGTTAGATGTGTGTATATAATGCATCCGAAAGATGTCTCAACGAACAAATGA 1360
TGGGCTGAAAAAGTTAAAAAAAACACTTACAATCTACACACATATATTACGTAGGCTTTCTACAGAGTTGCTTGTTTACT
                                                1328        1343
                                                 1330
                                                 1331
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ANSpromoter -> Restriction Map

```
            Sau3A I                                               Sau3A I
            Mbo I                                                 Mbo I
            Dpn I                    Mse I                        Dpn I
          Taq I                     Ase I              Mse I     Bcl I
          | |                       ||                 |         ||
GCCACCTACTTCGATCACTCGCTATCAATGTTATTAATGCCTTGTTGATTTTAATAGTTGATCAATAATAGTAAAATCTA  1440
CGGTGGATGAAGCTAGTGAGCGATAGTTACAATAATTACGGAACAACTAAAATTATCAACTAGTTATTATCATTTTAGAT
          | |                       ||                 |         ||
          1371                      1393               1411      1419
            1373                     1394                         1420
            1373                                                  1420
            1373                                                  1420

                                              Alu I
                                              Sac I
                                              HgiA I
                                              Bsp1286 I     Nla IV       BsmA I
                                         Mae I        Ase I Ban I      Taq I  Dde I
            BsmA I                 Mae III    Ban II  Mse I      Mse I    Hga I
            |                      |     |    ||      ||    |     |    | | |   |
TTCAAGGGTATAGTCTCCCGTTCACACTCATCGGGGTTACACTAGCGAGCTCCATTAATCGGTGCCTTAATCGAGACGCT  1520
AAGTTCCCATATCAGAGGGCAAGTGTGAGTAGCCCCAATGTGATCGCTCGAGGTAATTAGCCACGGAATTAGCTCTGCGA
            |                      |     |    ||      ||    |     |    | | |   |
            1453                   1476       1487    1495        1507   1515
                                         1482         1494  1501     1511    1519
                                              1487          1501       1513
                                              1487
                                              1487
                                               1488

                          Nla III
                         Sty I
                         Sec I
                         Nco I
                         Dsa I                              Sau3A I
                 Mae I  HinP I                              Mbo I
              Tth111 I  Hha I                               Dpn I
                                          Hae III           Alw I
           Nla III     Hae II             Hae I        Taq I            Mae I
           |  |  |     || ||              ||           |    |           |
AAGAACTATACCATGACCTAGTCAGCGCCATGGGACTGATGTAGGCCACACAATCTCGATGATCCGAAAACGCTAGAGTT  1600
TTCTTGATATGGTACTGGATCAGTCGCGGTACCCTGACTACATCCGGTGTGTTAGAGCTACTAGGCTTTTGCGATCTCAA
           |  |  |     || ||              ||           |    |           |
           1532        1544               1563         1576             1593
              1535      1545               1564             1581
                 1538   1545                                1581
                           1548                             1581
                           1548                             1581
                           1548
                           1548
                            1549

                 Sty I
                 Sec I
                 Nco I
                 Dsa I                                                Mae I
           Taq I  Nla III              EcoR V                       BsmA I
      Mae I  BsmA I   Mae III       BstU I                         Taq I      Drd I
      |    | |   ||   |             |  |                           | |   |      |
CAAGACCTAGTTCGAGACCATGGTCACGGTTTCAACCGCGATATCTCAACAATGCAATTTTTTTCGAGACTAGACAGACG  1680
GTTCTGGATCAAGCTCTGGTACCAGTGCCAAAGTTGGCGCTATAGAGTTGTTACGTTAAAAAAAGCTCTGATCTGTCTGC
      |    | |   ||   |             |  |                           | |   |      |
      1607   1614     1623          1637                           1664         1677
           1612  1619                  1640                          1666
                 1618                                                    1670
                 1618
                 1618
                 1618

                         Mae I                                  Mbo II
            Rsa I       Alu I   Mse I                           Bbv II    Nde I
            |          | |       |                              |         |
AATAAGTCTTGTGTACGATGGGTAGCTAGTGAATTAAAGGTAATCACTTTACTCGTGTTCACAAGAAGACCATTCATATG  1760
TTATTCAGAACACATGCTACCCATCGATCACTTAATTTCCATTAGTGAAATGAGCACAAGTGTTCTTCTGGTAAGTATAC
            |          | |       |                              |         |
            1693       1704      1714                           1745      1755
                         1706                                   1745
```

ANSpromoter -> Restriction Map

```
                    Alu I
                    Sac I                                  ScrF I
                    HgiA I                                 EcoR II
                    Bsp1286 I                          Mae III
             Dde I  Ban II      Mae I          Tth111 II   BstN I
             |      ||          |              |       |   |
AACATTTCGTGTTCTCAGACGAGCTCCTTTCGCTAGTTTGGTAAACTTGTTTGGTGTTACCTGGCTTATCATAGCCCACT  1840
TTGTAAAGCACAAGAGTCTGCTCGAGGAAAGCGATCAAACCATTTGAACAAACCACAATGGACCGAATAGTATCGGGTGA
             |      ||          |              |       |   |
             1774   1781        1793           1808        1820
                    1781                               1816
                    1781                                   1820
                    1781                                   1820
                     1782

                              Nla III
                             NspH I    Acc I
                             Nsp7524 I
                             ||        |
CAATTTGGCGAAAACATAACAATTGTTTCACATGCGAAGGTCTACACACCCATACTCGTGATAAAAACGGTTGCATTTAT  1920
GTTAAACCGCTTTTGTATTGTTAACAAAGTGTACGCTTCCAGATGTGTGGGTATGAGCACTATTTTTGCCAACGTAAATA
                             ||        |
                             1870
                             1870      1880
                              1871

                                                       Sau3A I
                                                       Mbo I
                                                       Dpn I
                           Tth111 II                 Taq I
            Taq I      Mae II                       Cla I            Bsr I
            |          |   |                        || |              |
TATTATTGCCTTTCGAGCAATTTACGTTGTTTGAAGTTTGTGTAAAAACAAAATCGATCTATAATTACTGACTGGAACGG  2000
ATAATAACGGAAAGCTCGTTAAATGCAACAAACTTCAAACACATTTTTGTTTTAGCTAGATATTAATGACTGACCTTGCC
            |          |   |                        || |              |
            1933       1944                         1973              1991
                           1948                      1974
                                                       1976
                                                       1976
                                                       1976

                Sau96 I                                                    Alu I
                Ava II                                                  Dde I
               PpuM I                                           Rsa I  Esp I
               EcoO109 I                 Alu I          Alu I         Alu I
                ||                       |              |   |         |||  |
TAATATCAAAACTGTTAGGACCTGTTCTTTTCGGCTTATAAGAGCTGAACTTATAAAAGCTGTACTTATAGGAGCTGAGC  2080
ATTATAGTTTTGACAATCCTGGACAAGAAAAGCCGAATATTCTCGACTTGAATATTTTCGACATGAATATCCTCGACTCG
                ||                       |              |   |         |||  |
                2017                     2043           2058          2073
                2017                                        2062       2074
                 2018                                                   2075
                 2018                                                      2078

                                               Alu I
                                            Mse I       Xmn I
                                            |  |        |
TGAACTGAACTTATAAGAGTTGAACTGAACTTATAAGGGCTGAAATTAAGCTGTAAAGAACAGGTTCTTATTTCGCCTGA  2160
ACTTGACTTGAATATTCTCAACTTGACTTGAATATTCCCGACTTTAATTCGACATTTCTTGTCCAAGAATAAAGCGGACT
                                            |  |        |
                                            2126        2138
                                               2129

                                                                        Mme I
                 Fok I          Mnl I                          Ple I    Hinf I
   Hinf I    Mme I   Mbo II                                    Hinf I  Taq I
   |          |  |   |      |                              |       | |  |
TTTGAATCACTAAAGTTGGATGAAGATAGAGGCGAGAAACGCTCCCCTTTCCACAGTTTGACTCTACTCGAATCCGACCA  2240
AAACTTAGTGATTTCAACCTACTTCTATCTCCGCTCTTTGCGAGGGGAAAGGTGTCAAACTGAGATGAGCTTAGGCTGGT
   |          |  |   |      |                              |       | |  |
   2164       2175   2182                                  2220    2228
                 2178       2189                           2220      2230
                                                                        2233
```

ANSpromoter -> Restriction Map

```
                                                                               Xca I
                                                                        Fnu4H I
                                                                        Bbv I
                             Mnl I
     Fok I              Bsm I    Nla III   HgiE II            Nla III  Alu I   Acc I
     |                  |    |  |         |                   |         ||     |
AAATTGGATGTAAATTAGAAGTGAGCATTCCTCATGCCAACCACCGTTCTTGGTCGTGAAAACATGATTTGTAGCTGCTG  2320
TTTAACCTACATTTAATCTTCACTCGTAAGGAGTACGGTTGGTGGCAAGAACCAGCACTTTTGTACTAAACATCGACGAC
     |   •         •    |    |  |      •  |      •         •  |      •  ||     |
     2246               2265   2273      2283                2303      2313   2320
                             2270                                       2314
                                                                        2314
                                                                               2320

                                  Mme I   Mse I
                                  |       |
TATACTTCCCAAACCCGTGATAATCTGCCACACTTCCAACACTTAATTGATTTTTATCAAAATTCCTACAGTTTTTTGGT  2400
ATATGAAGGGTTTGGGCACTATTAGACGGTGTGAAGGTTGTGAATTAACTAAAAATAGTTTTAAGGATGTCAAAAAACCA
         •         •         •    |    •  |      •         •         •         •
                                  2355    2363
                                  Mae III    HinC II
                                  |          |
TTTACTCCTAAACTATGCTGTCTTTTTACAAGTTGTTACACCTTTGTCAACAACTTTATGCTTTATTATATTTCTTATAT  2480
AAATGAGGATTTGATACGACAGAAAAATGTTCAACAATGTGGAAACAGTTGTTGAAATACGAAATAATATAAAGAATATA
         •         •         •    |    •     |   •         •         •         •
                                  2435       2446
                                                                   Mae I
                                      Mse I               Mnl I   Xba I
                                      |                   |       ||
AAAGACCATATAACTTCCCTACACTAATGCCACAACACTTAAAAGCATACAACACAAACCTCATAATCTAGA  2552
TTTCTGGTATATTGAAGGGATGTGATTACGGTGTTGTGAATTTTCGTATGTTGTGTTTGGAGTATTAGATCT
         •         •         •        |•         •        |•      || •
                                      2519                2539    2547
                                                                   2548
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CLAIMS:

1. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a cytochrome b_5 (Cyt b_5) molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent or homologue thereof.

2. An isolated nucleic acid molecule according to claim 1 wherein the Cyt b_5 modulates or otherwise facilitates activity of a cytochrome P450 molecule (Cyt P450).

3. An isolated nucleic acid molecule according to claim 1 or 2 wherein the Cyt P450 comprises the amino acid sequence
(F/P/W/L/Y)(G/S/C/N/H/E)X(G/D/A/E)X
(R/H/S/K/T) XCX_a(G/A) wherein X is any amino acid and X_a is selected from V, M, A, L, I, P, F or T and wherein the residues in parentheses represent alternatives of a single position.

4. An isolated nucleic acid molecule according to claim 3 wherein the Cyt P450 molecule is involved in or otherwise associated with the direct or indirect hydroxylation of a flavonoid compound.

5. An isolated nucleic acid molecule according to claim 4 wherein the Cyt P450 is flavonoid 3',5'-hydroxylase (F3',5'H).

6. An isolated nucleic acid molecule according to claim 4 wherein the Cyt P450 is flavonoid 3'-hydroxylase (F3'H).

7. An isolated nucleic acid molecule according to any one of claims 1 to 6 wherein the Cyt b_5 comprises the amino acid sequence:

Y K A S D D S E L E L N L V T D S I K E P N

or an amino acid sequence having at least 70% similarity thereto.

8. An isolated nucleic acid molecule according to claim 7 wherein Cyt b_5 comprises the amino acid sequence:

$$[X_1X_2 \ldots X_n]KE[X_1',X_2' \ldots X_{n1}']$$
$$F[X_1'', X_2'' \ldots X_{n2}'']$$
$$Y K A S D D S E L E L N L V T D S I K E P N D S I K$$
$$E P N [X_1''', X_2''' \ldots X'''_{n3}] E D P K P Y L T F V E S$$

wherein $[X_1, X_2 \ldots X_n]$, $[X_1', X_2' \ldots X'_{n1}]$, $[X''_1, X_2'' \ldots X''_{n2}]$ and $[X_1''', X_2''' \ldots X_{n2}''']$ are amino acid sequences of any amino acid residues up to n, n_1, n_2 and n_3 amino acid residues in length wherein n, n_1, n_2 and n_3 may be the same or different and each is from about 1 to about 200.

9. An isolated nucleic acid molecule according to any one of claims 1 to 8 wherein the Cyt b_5 comprises the amino acid sequence substantially as set forth in <400>2 or an amino acid sequence having at least about 30% similarity thereto.

10. An isolated nucleic acid molecule according to any one of claims 1 to 9comprising the nucleotide sequence substantially as set forth in <400>1, or a nucleotide sequence having at least about 30% similarity thereto or a nucleotide sequence capable of hybridizing to <400>1 under low stringency conditions at 42°C.

11. A genetic construct in single or multicistronic form wherein at least one cistron encodes a Cyt b_5 or a mutant part, fragment or portion thereof or a functional and/or structural equivalent of homologue thereof; the genetic construct optionally further comprising cistrons encoding one or both of a Cyt P450 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent of homologue thereof; and/or a reductase or other associated protein.

12. A genetic construct according to claim 11 wherein the Cyt b_5 modulates or

otherwise facilitates activity of the Cyt P450 encoded by the same genetic construct or a Cyt P450 on another genetic construct or encoded by the genome of a host cell.

13. A genetic construct according to claim 11 or 12 wherein the Cyt P450 molecule comprises the amino acid sequence
(F/P/W/L/Y)(G/S/C/N/H/E)X(G/D/A/E)X
(R/H/S/K/T) XCX_a(G/A) wherein X is any amino acid and X_a is selected from V, M, A, L, I, P, F or T and wherein the residues in parentheses represent alternatives of a single position.

14. A genetic construct according to claim 13 wherein the Cyt P450 molecule is involved in or otherwise associated with the direct or indirect hydroxylation of a flavonoid compound.

15. A genetic construct according to claim 14 wherein the Cyt P450 molecule is F3',5'H.

16. A genetic construct according to claim 14 wherein the Cyt P450 molecule is F3'H.

17. A genetic construct according to any one of claims 11 to 16 wherein the Cyt b_5 comprises the amino acid sequence:

Y K A S D D S E L E L N L V T D S I K E P N

or an amino acid sequence having at least 70% similarity thereto.

18. A genetic construct according to claim 17 wherein the Cyt b_5 comprises the amino acid sequence:

$$[X_1X_2 \ldots X_n]KE[X_1',X_2' \ldots X_{n1}']$$
$$F[X_1'', X_2'' \ldots X_{n2}'']$$

Y K A S D D S E L E L N L V T D S I K E P N D S I K

E P N [X_1"', X_2"' ... X"'$_{n3}$] E D P K P Y L T F V E S

wherein [X_1, X_2 ... X_n], [X_1', X_2' ... X'$_{n1}$], [X"$_1$, X_2" ... X"$_{n2}$] and [X_1"', X_2'" ... X_{n2}"'] are amino acid sequences of any amino acid residues up to n, n_1, n_2 and n_3 amino acid residues in length wherein n, n_1, n_2 and n_3 may be the same or different and each is from about 1 to about 200.

19. A genetic construct according to any oen of claims 11 to 18 claim 17 or 18 wherein the Cyt b_5 comprises the amino acid sequence substantially as set forth in <400>2 or an amino acid sequence having at least about 30% similarity thereto.

20. A genetic construct according to any one of claims 11 to 19 wherein the Cyt b_5 is encoded by a nucleotide sequence substantially as set forth in <400>1, or a nucleotide sequence having at least about 30% similarity thereto or a nucleotide sequence capable of hybridizing to <400>1 under low stringency conditions at 42°C.

21. A transgenic plant or part thereof or cells therefrom comprising genetic material encoding a Cyt b_5 molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent or homologue thereof.

22. A transgenic plant or part thereof or cells therefrom according to claim 21 wherein
the Cyt b_5 modulates or otherwise facilitates activity of a Cyt P450.

23. A transgenic plant or part thereof or cells therefrom according to claim 21 or 22 wherein the Cyt P450 molecule comprises the amino acid sequence (F/P/W/L/Y)(G/S/C/N/H/E)X(G/D/A/E)X(R/H/S/K/T) XCX_a(G/A) wherein X is any amino acid and X_a is selected from V, M, A, L, I, P, F or T and wherein the residues in parentheses represent alternatives of a single position.

24. A transgenic plant or part thereof or cells therefrom according to claim 23 wherein the Cyt P450 molecule is involved in or otherwise associated with the direct or indirect hydroxylation of a flavonoid compound.

25. A transgenic plant or part thereof or cells therefrom according to claim 24 wherein the Cyt P450 is F3',5'H.

26. A transgenic plant or part thereof or cells therefrom according to claim 24 wherein the Cyt P450 is F3'H.

27. A transgenic plant or part thereof or cells therefrom according to any one of claims 21 to 26 wherein the Cyt b_5 comprises the amino acid sequence:

Y K A S D D S E L E L N L V T D S I K E P N

or an amino acid sequence having at least 70% similarity thereto.

28. A transgenic plant or part thereof or cells therefrom according to claim 27 wherein Cyt b_5 comprises the amino acid sequence:

$[X_1X_2 \ldots X_n]KE[X_1',X_2' \ldots X_{n1}']$

$F[X_1'', X_2'' \ldots X_{n2}'']$

Y K A S D D S E L E L N L V T D S I K E P N D S I K

E P N $[X_1''', X_2''' \ldots X'''_{n3}]$ E D P K P Y L T F V E S

wherein $[X_1, X_2 \ldots X_n]$, $[X_1', X_2' \ldots X'_{n1}]$, $[X''_1, X_2'' \ldots X''_{n2}]$ and $[X_1''', X_2''' \ldots X_{n2}''']$ are amino acid sequences of any amino acid residues up to n, n_1, n_2 and n_3 amino acid residues in length wherein n, n_1, n_2 and n_3 may be the same or different and each is from about 1 to about 200.

29. A transgenic plant or part thereof or cells therefrom according to claims 21 to 28 wherein the Cyt b_5 comprises the amino acid sequence substantially as set forth in <400>2 or an amino acid sequence having at latest about 30% similarity thereto.

30. A transgenic plant or part thereof or cells therefrom according to any one of claims 21 to 29 or 28 or 29 comprising the nucleotide sequence substantially as set forth in <400>1, or a nucleotide sequence having at least about 30% similarity thereto or a nucleotide sequence capable of hybridizing to <400>1 under low stringency conditions at 42°C.

31. A method of expressing a nucleotide sequence encoding a Cyt P450 or a functional derivative, homologue or equivalent thereof in a plant or cells of a plant, said method comprising introducing into said plant or cells of said plant a genetic construct in single or multicistronic form wherein at least one cistron encodes a Cyt b_5 or a mutant part, fragment or portion thereof or a functional and/or structural equivalent of homologue thereof; the genetic construct optionally further comprising cistrons encoding one or both of a Cyt P450 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent of homologue thereof; and/or a reductase or other associated protein.

32. A method according to claim 31 wherein the Cyt b_5 modulates or otherwise facilitates activity of the Cyt P450 encoded by the same genetic construct or a Cyt P450 on another genetic construct or encoded by the genome of a host cell.

33. A method according to claim 31 or 32 wherein the Cyt P450 molecule comprises the amino acid sequence (F/P/W/L/Y)(G/S/C/N/H/E)X(G/D/A/E)X (R/H/S/K/T) XCX$_a$(G/A) wherein X is any amino acid and X_a is selected from V, M, A, L, I, P, F or T and wherein the residues in parentheses represent alternatives of a single position.

34. A method according to claim 33 wherein the Cyt P450 molecule is involved in or otherwise associated with the direct or indirect hydroxylation of a flavonoid compound.

35. A method according to claim 34 wherein the Cyt P450 molecule is

F3',5'H.

36. A method according to claim 34 where the Cyt P450 molecule is F3'H.

37. A method according to any one of claims 31 to 36 wherein the Cyt b_5 comprises the amino acid sequence:

Y K A S D D S E L E L N L V T D S I K E P N

or an amino acid sequence having at least 70% similarity thereto.

38. A method according to claim 37 where the Cyt b_5 comprises the amino acid sequence:

$[X_1X_2 \ldots X_n]KE[X_1',X_2' \ldots X_{n1}']$

$F[X_1'', X_2'' \ldots X_{n2}'']$

Y K A S D D S E L E L N L V T D S I K E P N D S I K

E P N $[X_1''', X_2''' \ldots X'''_{n3}]$ E D P K P Y L T F V E S

wherein $[X_1, X_2 \ldots X_n]$, $[X_1', X_2' \ldots X'_{n1}]$, $[X''_1, X_2'' \ldots X''_{n2}]$ and $[X_1''', X_2''' \ldots X_{n2}''']$ are amino acid sequences of any amino acid residues up to n, n_1, n_2 and n_3 amino acid residues in length wherein n, n_1, n_2 and n_3 may be the same or different and each is from about 1 to about 200.

39. A method according to any one of claims 31 to 38 where the Cyt b_5 comprises the amino acid sequence substantially as set forth in <400>2 or an amino acid sequence having at latest about 30% similarity thereto.

40. A method according to any one of claims 31 to 39 where the Cyt b_5 is encoded by a nucleotide sequence substantially as set forth in <400>1, or a nucleotide sequence having at least about 30% similarity thereto or a nucleotide sequence capable of hybridizing to <400>1 under low stringency conditions at 42°C.

41. Flowers cut or severed from a plant according to any one of claims 21 to

30.

42. Reproductive parts of a plant according to any one of claims 21 to 30.

43. Use of a genetic construct comprising a nucleotide sequence encoding a Cyt b_5 or a mutant part, fragment or portion thereof or a functional and/or structural equivalent or homologue thereof in the manufacture of a plant or cells of a plant in which said Cyt b_5 or a mutant part, fragment or portion thereof or a functional and/or structural equivalent or homologue thereof enhances, modulates or otherwise facilitates expression of genetic material encoding a Cyt P450 or activity of a Cyt P450.

44. Use according to claim 43 wherein the Cyt P450 molecule comprises the amino acid sequence (F/P/W/L/Y)(G/S/C/N/H/E)X(G/D/A/E)X(R/H/S/K/T) XCX_a(G/A) wherein X is any amino acid and X_a is selected from V, M, A, L, I, P, F or T and wherein the residues in parentheses represent alternatives of a single position.

45. Use according to claim 45 wherein the Cyt P450 molecule is involved in or otherwise associated with the direct or indirect hydroxylation of a flavonoid compound.

46. Use according to claim 46 wherein the Cyt P450 is flavonoid 3',5'-hydroxylase F3',5'H.

47. Use according to claim 46 wherein the Cyt P450 is F3'H.

48. Use according to any one of claims 44 to 47 wherein the Cyt b_5 comprises the amino acid sequence:

Y K A S D D S E L E L N L V T D S I K E P N

or an amino acid sequence having at least 70% similarity thereto.

49. Use according to claim 49 wherein Cyt b_5 comprises the amino acid sequence:

$$[X_1X_2 \ldots X_n]KE[X_1',X_2' \ldots X_{n1}']$$
$$F[X_1'', X_2'' \ldots X_{n2}'']$$

Y K A S D D S E L E L N L V T D S I K E P N D S I K

E P N $[X_1''', X_2''' \ldots X'''_{n3}]$ E D P K P Y L T F V E S

wherein $[X_1, X_2 \ldots X_n]$, $[X_1', X_2' \ldots X'_{n1}]$, $[X''_1, X_2'' \ldots X''_{n2}]$ and $[X_1''', X_2''' \ldots X_{n2}''']$ are amino acid sequences of any amino acid residues up to n, n_1, n_2 and n_3 amino acid residues in length wherein n, n_1, n_2 and n_3 may be the same or different and each is from about 1 to about 200.

50. Use according to any one of claims 43 to go wherein the Cyt b_5 comprises the amino acid sequence substantially as set forth in <400>2 or an amino acid sequence having at latest about 30% similarity thereto.

51. Use according to any one of claims 43 to 50 comprising the nucleotide sequence substantially as set forth in <400>1, or a nucleotide sequence having at least about 30% similarity thereto or a nucleotide sequence capable of hybridizing to <400>1 under low stringency conditions at 42°C.

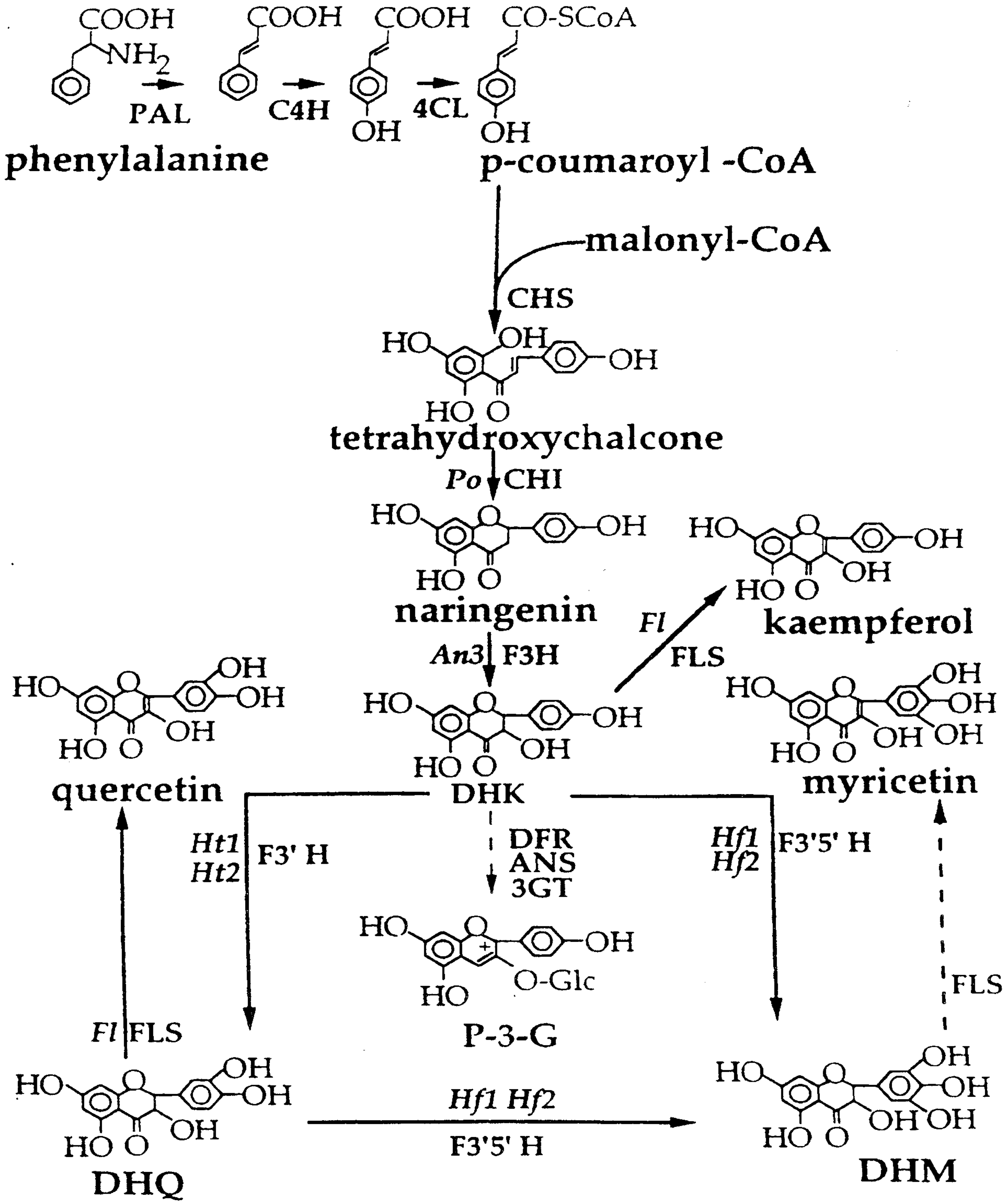
COOH
NH2
PAL
C4H
COOH
COOH
4CL
OH
CO-SCoA
OH
phenylalanine
p-coumaroyl -CoA
malonyl-CoA
CHS
tetrahydroxychalcone
Po CHI
naringenin
Fl
FLS
kaempferol
An3 F3H
quercetin
DHK
myricetin
Ht1
Ht2
F3' H
DFR
ANS
3GT
Hf1
Hf2
F3'5' H
O-Glc
P-3-G
FLS
Fl FLS
Hf1 Hf2
F3'5' H
DHQ
DHM

Figure 1a

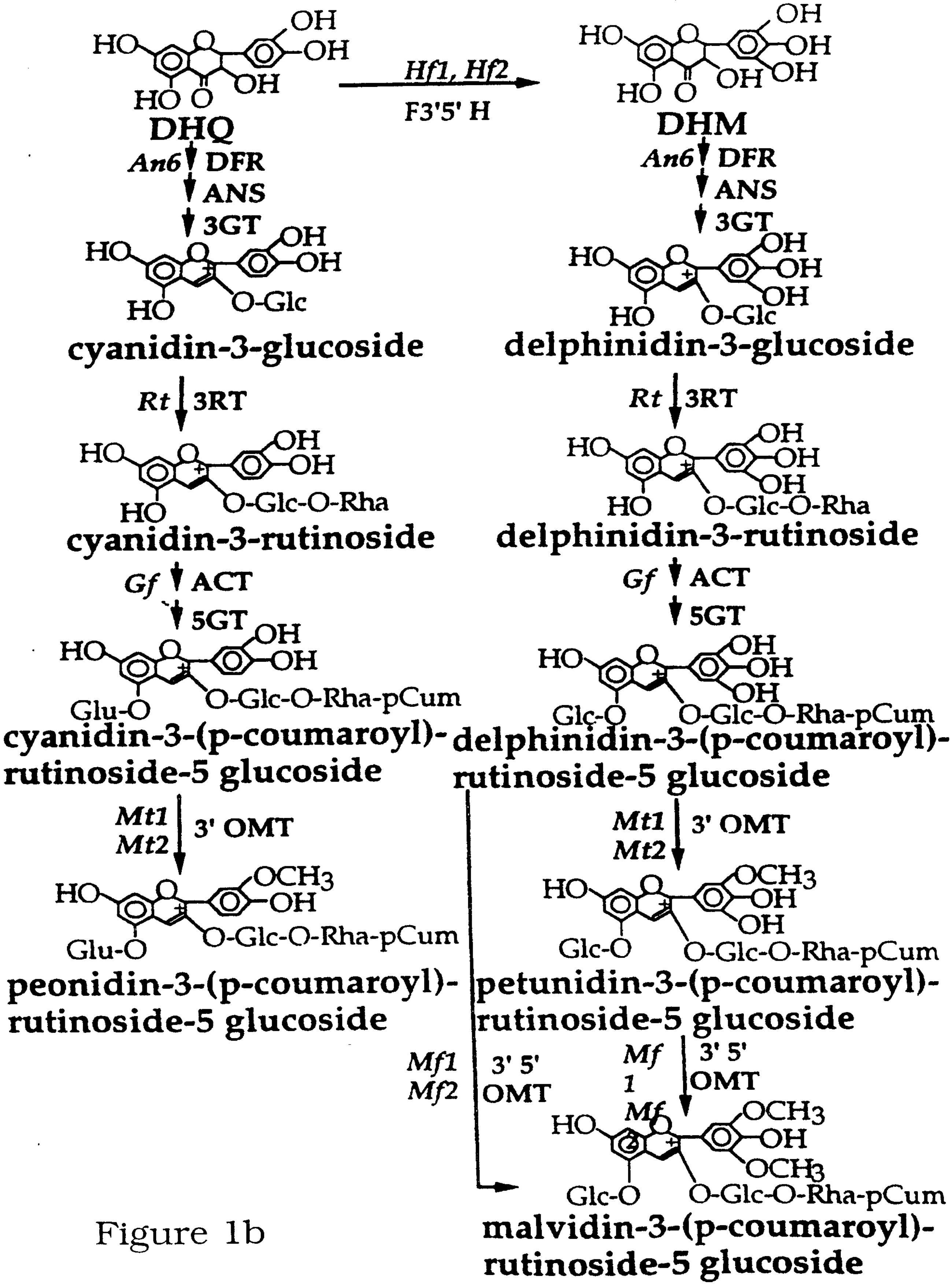
Hf1, Hf2
F3'5' H
DHQ
DHM
An6
DFR
ANS
3GT
cyanidin-3-glucoside
delphinidin-3-glucoside
Rt
3RT
cyanidin-3-rutinoside
delphinidin-3-rutinoside
Gf
ACT
5GT
cyanidin-3-(p-coumaroyl)-rutinoside-5 glucoside
delphinidin-3-(p-coumaroyl)-rutinoside-5 glucoside
Mt1
Mt2
3' OMT
peonidin-3-(p-coumaroyl)-rutinoside-5 glucoside
petunidin-3-(p-coumaroyl)-rutinoside-5 glucoside
Mf1
Mf2
3' 5' OMT
malvidin-3-(p-coumaroyl)-rutinoside-5 glucoside

Figure 1b

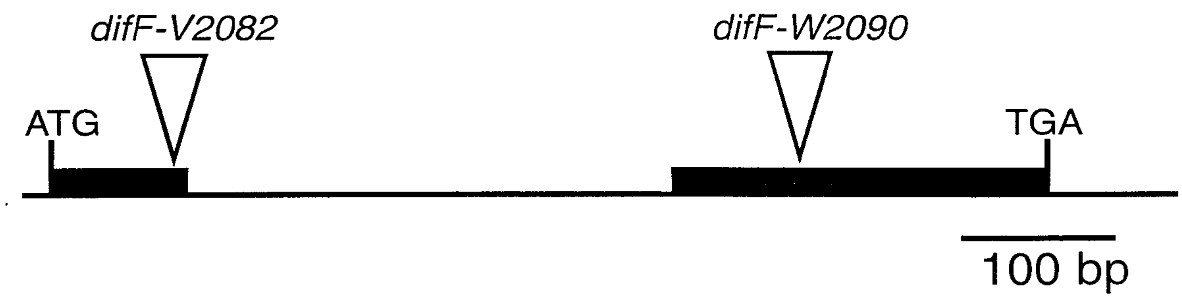

Figure 2a

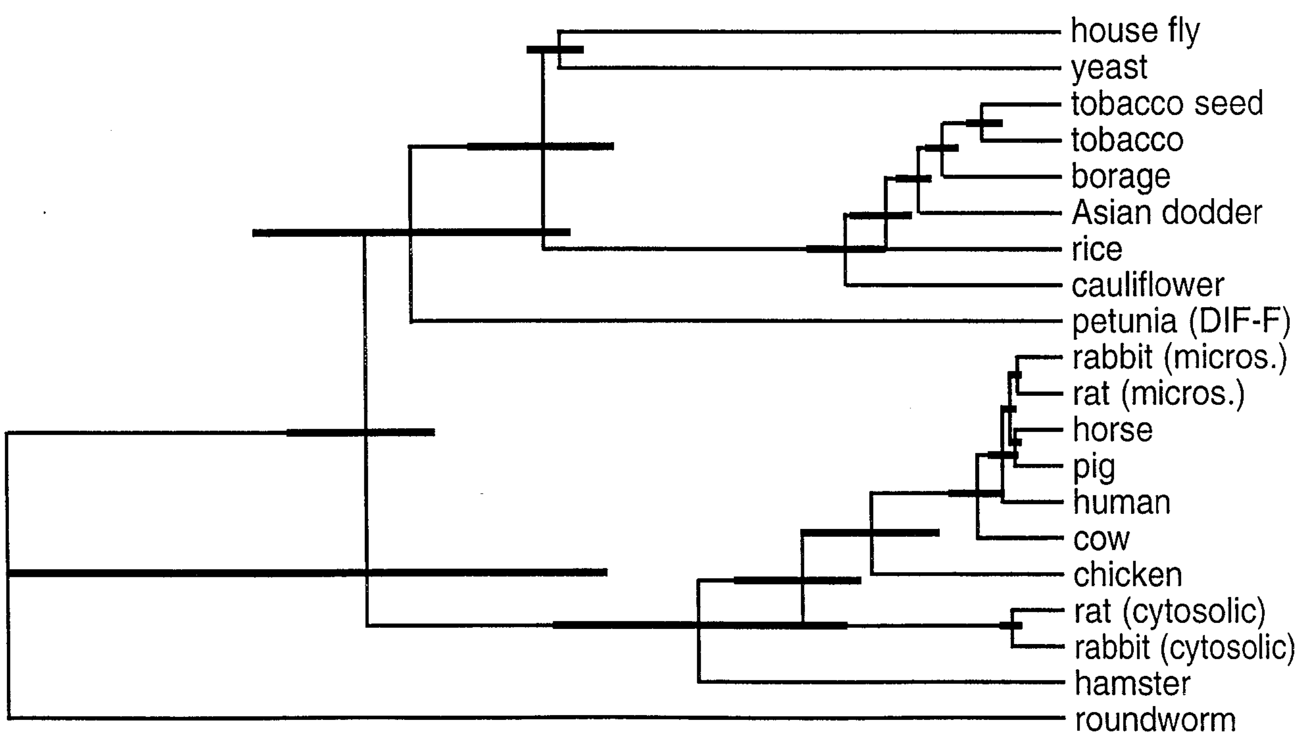

Figure 2b

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rabbit   MAAQSDKDVKYYTLEEIKKHNHSKSTWLILHHKVYDLTKF   40
petunia  M----DKQ-RVFTLSQVAEHKSKQDCWIIINGRVVDVTKF   35
tobacco  MIIMGGET-KVFTLAEVSQHNNAKDCWLVISGKVYDVTKF   39
yeast    M------P-KVYSYQEVAEHNGPQNFWIIIDDKVYDVSQF   33

cow      LEEHPGGEEVLREQAGGDATENFEDVGHSTDARELSKTFI   80
rabbit   LEEHPGGEEVLREQAGGDATENFEDVGHSTDARELSKTFI   80
petunia  LEEHPGGEEVLIESAGKDATKEFQDIGHSKAAKNLLFKYQ   75
tobacco  LDDHPGGDEVLLSATGKDATDDFEDVGHSSSARAMLDEYY   79
yeast    KDEHPGGDEIIMDLGGQDATESFVDIGHSDEALRLLKGLY   73

cow      IGELH-----PDDRSKITKPSESI--------ITTIDSNP  107
rabbit   IGELH-----PDDRSKLSKPMEP-----------------   98
petunia  IGYLQGYKASDDSELELNLVTDSIKEPNKAKEMKAYVIKE  115
tobacco  VGDIDS--ATIPTKTKYTPPNQPHYNQDKTSE---FVV--  112
yeast    IGDVD----KTSERVSVEKVSTSE-NQSKGSG--------  100

cow      SWWTNW------LIPAISALFVALIYHLYTSEN-        134
rabbit   ----------------------------------         98
petunia  DPKPKYLTFVEYLLPFLAAAFYLYYRYLTGALQF        149
tobacco  ----KLLQFLVPLI-ILGVAFGI--RFYTKQSSA        139
yeast    ----TLVVILAILM--LGVAY-----YL---LNE        120
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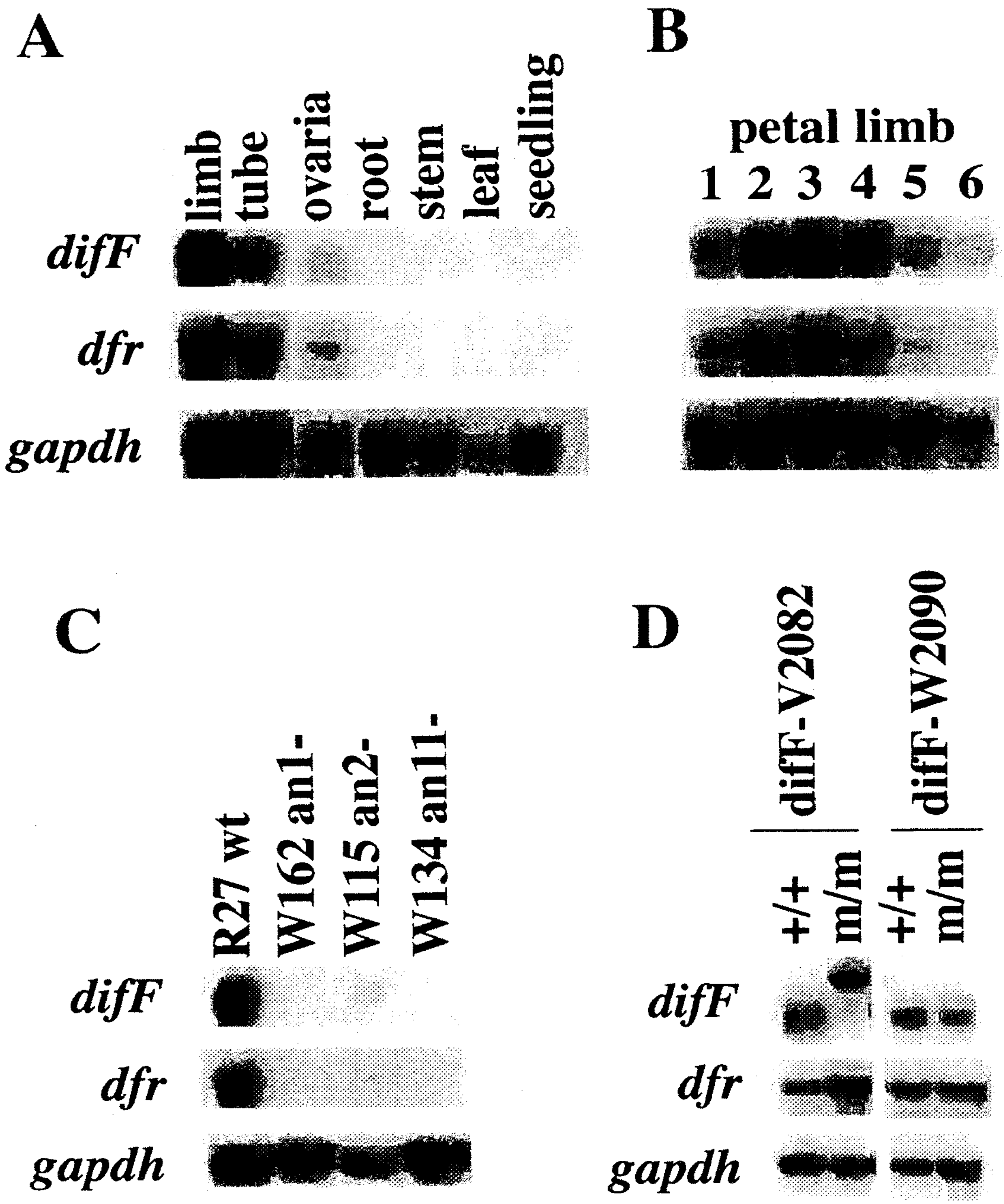
Figure 2c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limb
tube
ovaria
root
stem
leaf
seedling
difF
dfr
gapdh
B
petal limb
1 2 3 4 5 6
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R27 wt
W162 an1-
W115 an2-
W134 an11-
difF
dfr
gapdh
D
difF-V2082
difF-W2090
+/+
m/m
+/+
m/m
difF
dfr
gapdh

Figure 3

$hf1^{+} hf2^{-} rt^{+} difF^{m}$

$hf1^{+} hf2^{-} rt^{-} difF^{m}$

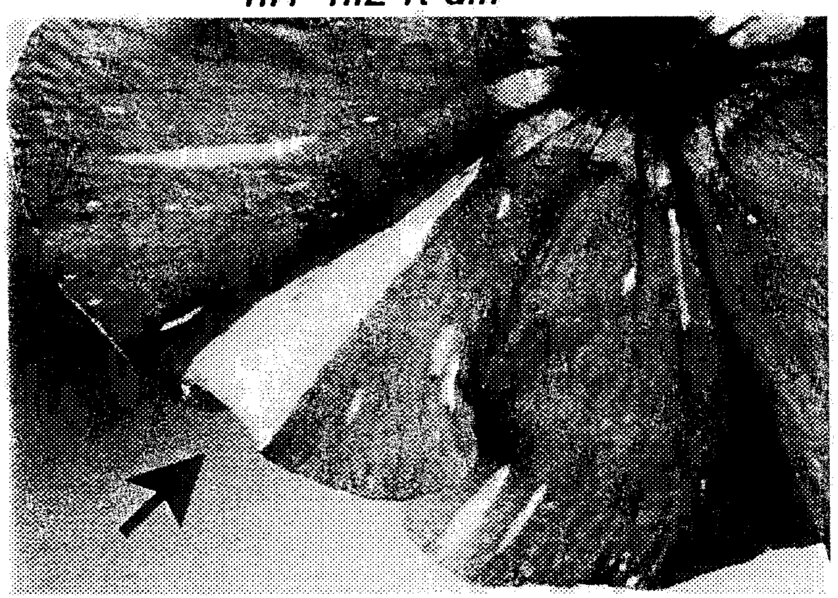


Figure 4a

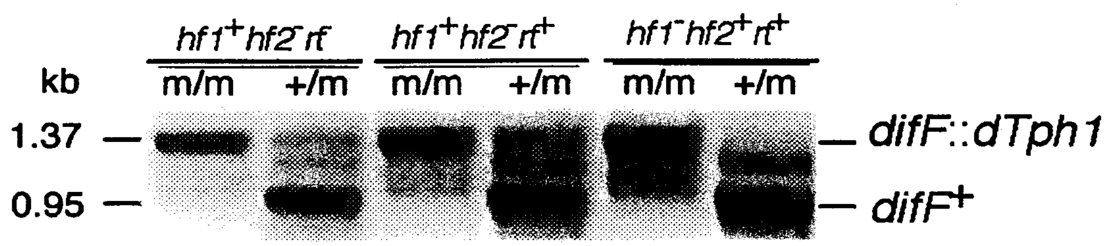
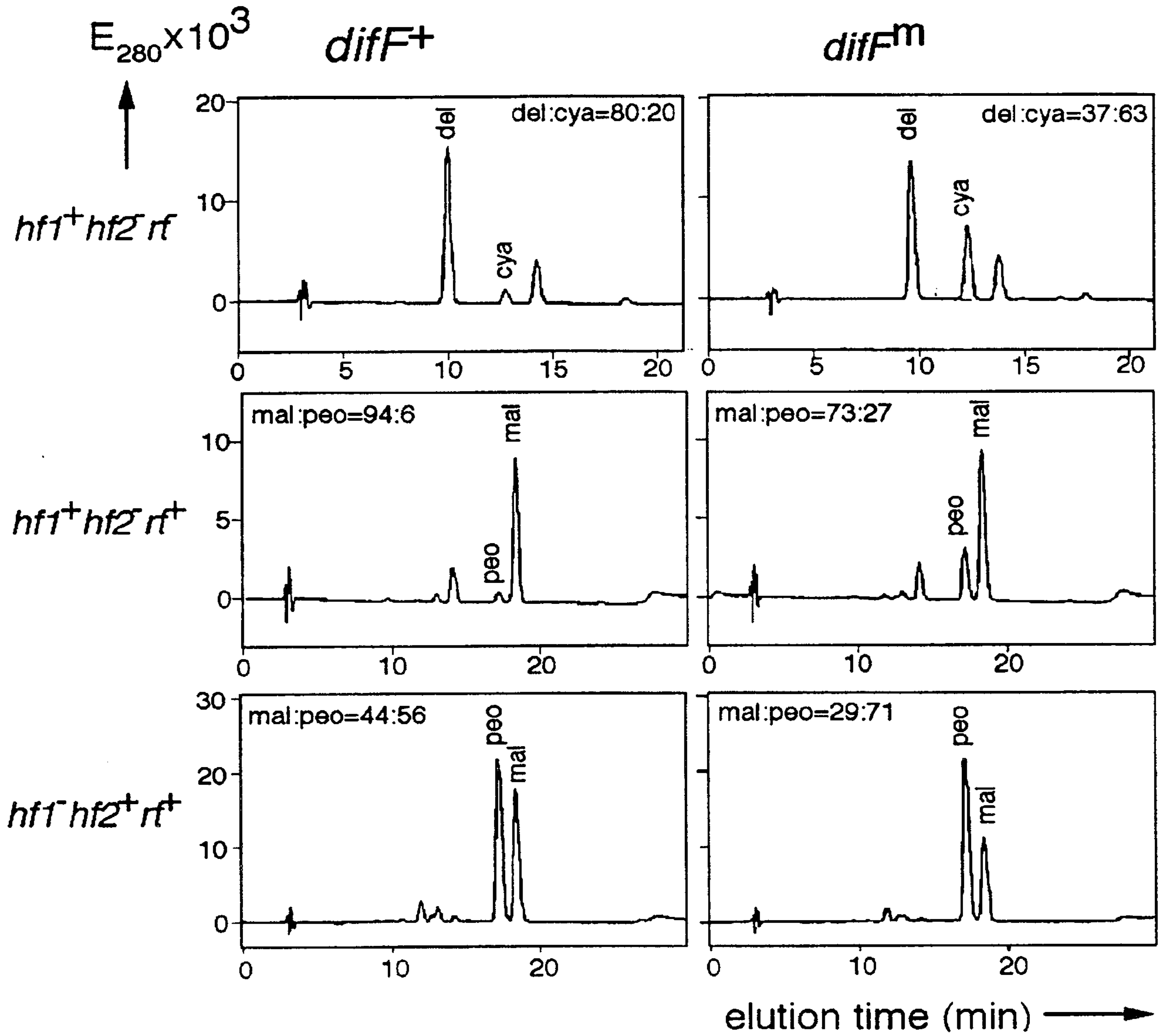

hf1+ hf2- rf-
hf1+ hf2- rf+
hf1- hf2+ rf+
kb
m/m
+/m
m/m
+/m
m/m
+/m
1.37
0.95
difF::dTph1
difF+

Figure 4b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difF+
difFm
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hf1+hf2-rf+
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mal:peo=94:6
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mal:peo=44:56
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del
cya
mal
peo
elution time (min)

Figure 4c

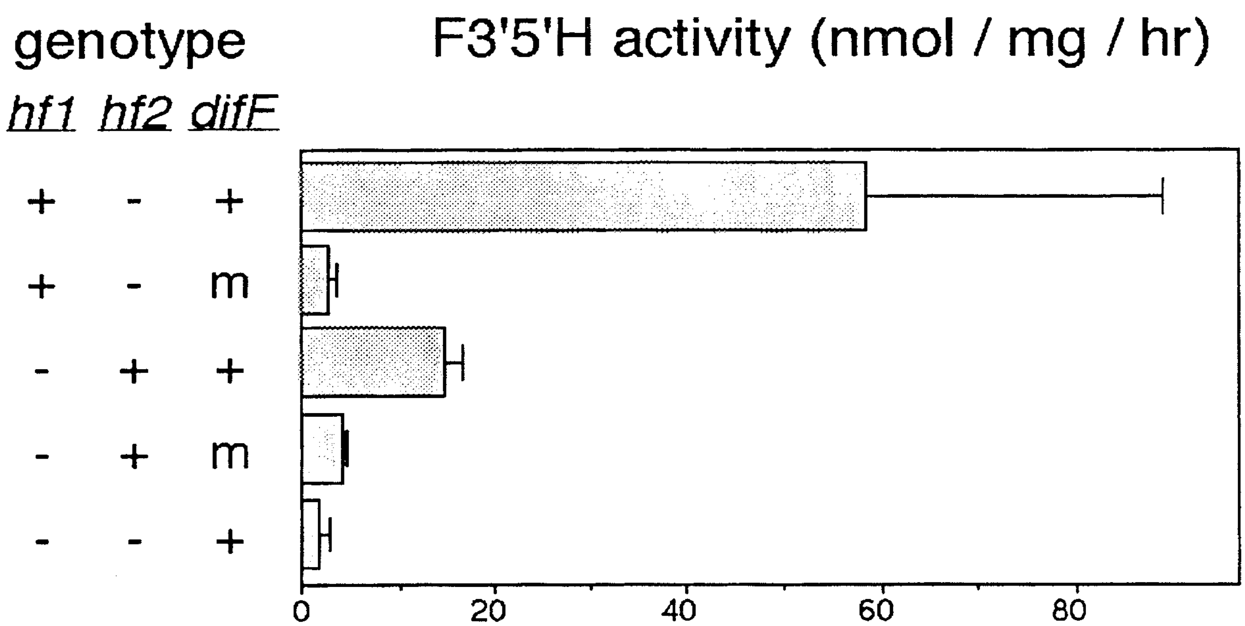

Figure 4d

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TCAATGGCAGAGTAGTAGATGTAACAAAGTTCTTGGAAGAACATCCTGGAGGAGAAGAAGTGTTGATTGAATCAG   150
  x2324
 N  G  R  V  V  D  V  T  K  F  L  E  E  H  P  G  G  E  E  V  L  I  E  S  A   (50)

CAGGAAAGGATGCAACTAAAGAGTTTCAAGATATTGGACATAGTAAAGCTGCCAAGAACTTGCTTTTCAAATACC   225
 G  K  D  A  T  K  E  F  Q  D  I  G  H  S  K  A  A  K  N  L  L  F  K  Y  Q   (75)

AAATTGGATATCTTCAAGGTTACAAAGCCTCAGATGATTCTGAACTTGAACTCAACTTAGTCACTGATTCCATCA   300
                     X2325
 I  G  Y  L  Q  G  Y  K  A  S  D  D  S  E  L  E  L  N  L  V  T  D  S  I  K   (100)

AAGAACCAAATAAGGCCAAAGAAATGAAAGCTTATGTTATCAAAGAAGATCCTAAGCCAAAGTATCTGACTTTTG   375)
 E  P  N  K  A  K  E  M  K  A  Y  V  I  K  E  D  P  K  P  K  Y  L  T  F  V   (125)

TTGAGTACTTATTGCCCTTCTTGGCTGCTGCCTTCTACCTCTATTATCGCTATCTCACTGGAGCTCTCCAGTTTT   450
 E  Y  L  L  P  F  L  A  A  A  F  Y  L  Y  Y  R  Y  L  T  G  A  L  Q  F  *   (149)

GAGCTCAGAGAACAAAGGATTACACTACATGATTATTGTCAGTATATTCTCACTGGAGCTATCGCATTGTTTGAA   525
CCTTAGAAGATACTTGGTGATTCTGGAAAAGTGTTTTCTTTATTTATTTTAATCTTCAAAGAAAGCTGGAGTTAC   600
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```

Figure 5

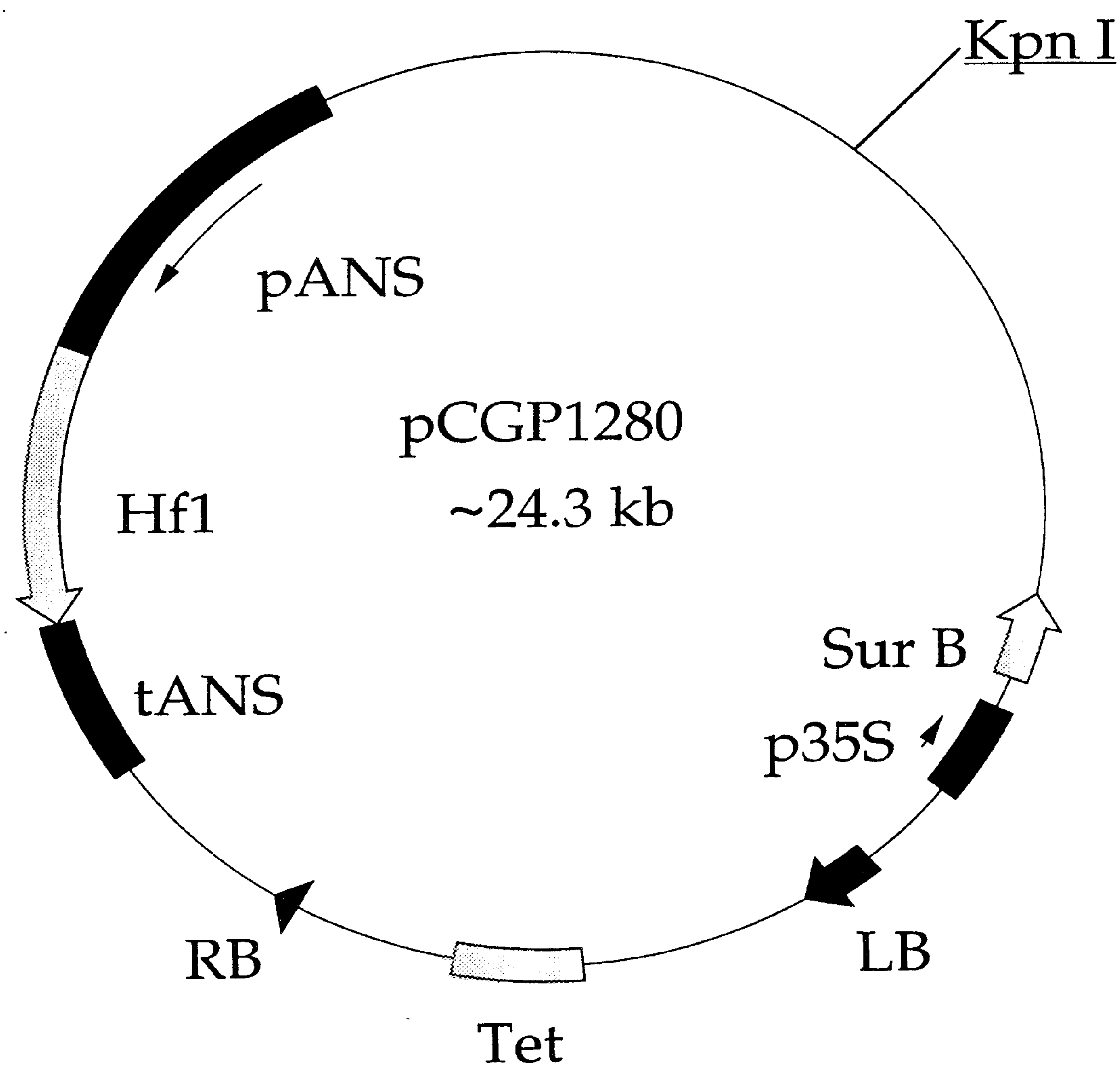


Figure 6a

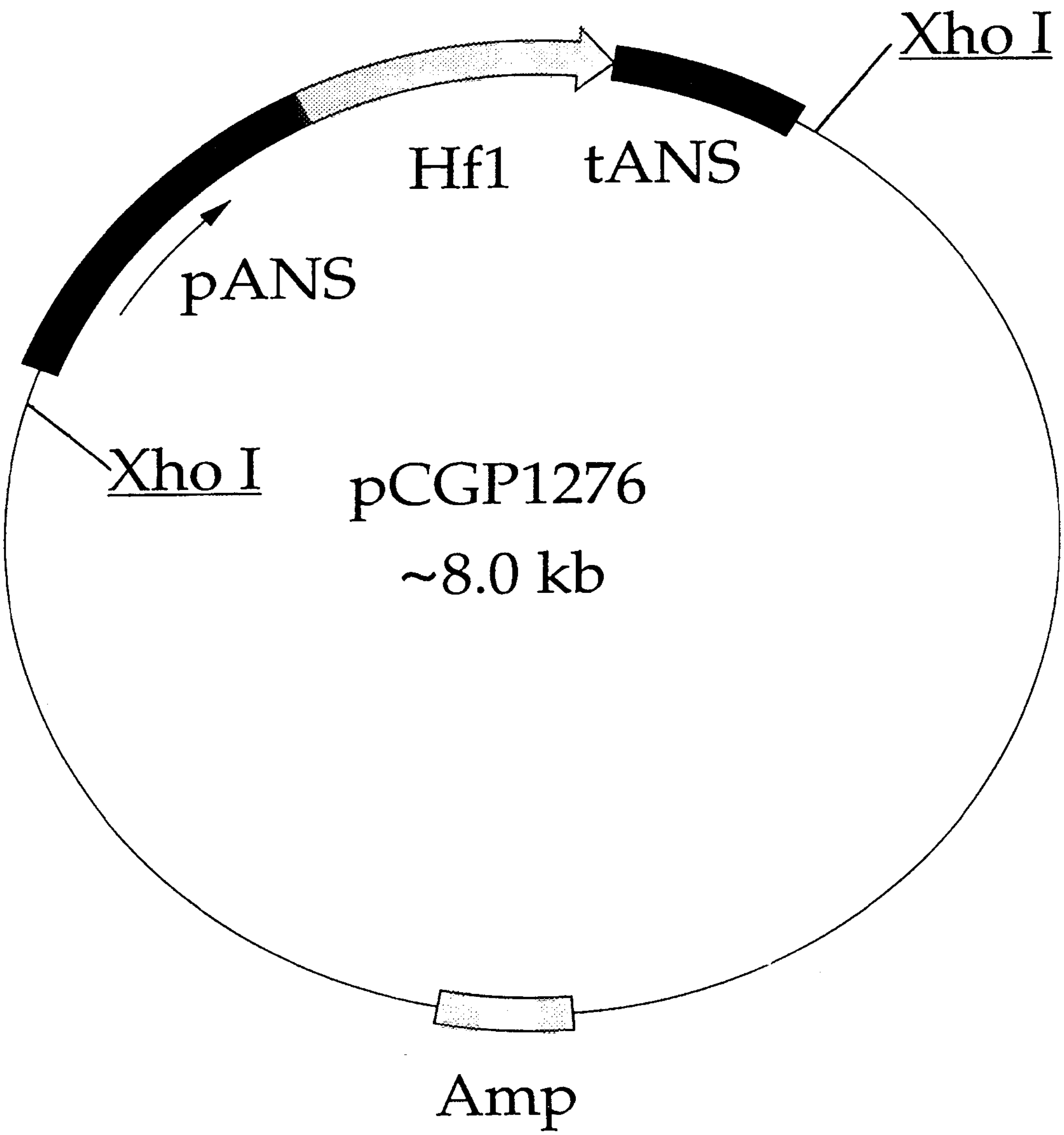


Figure 6b

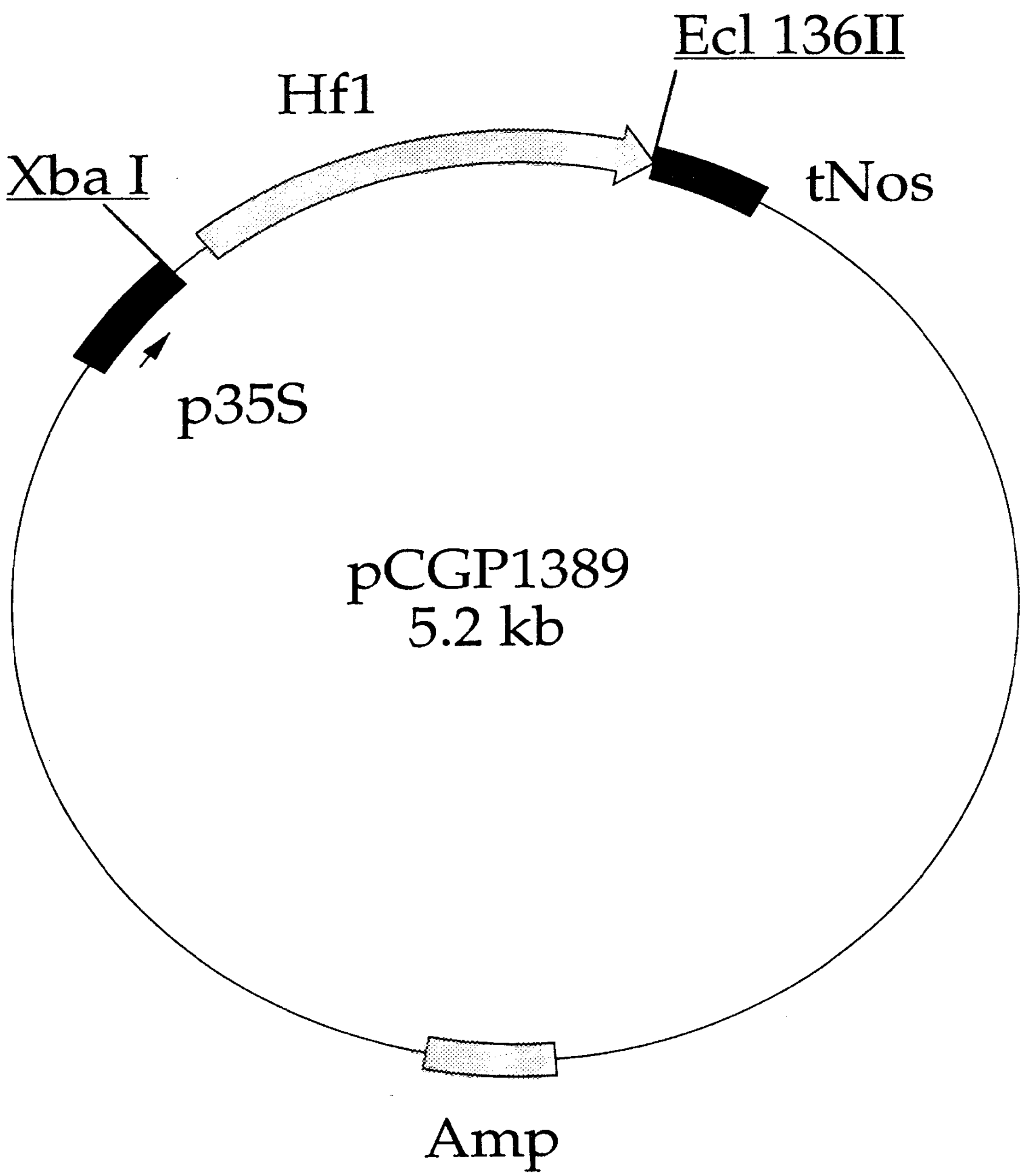


Figure 6c

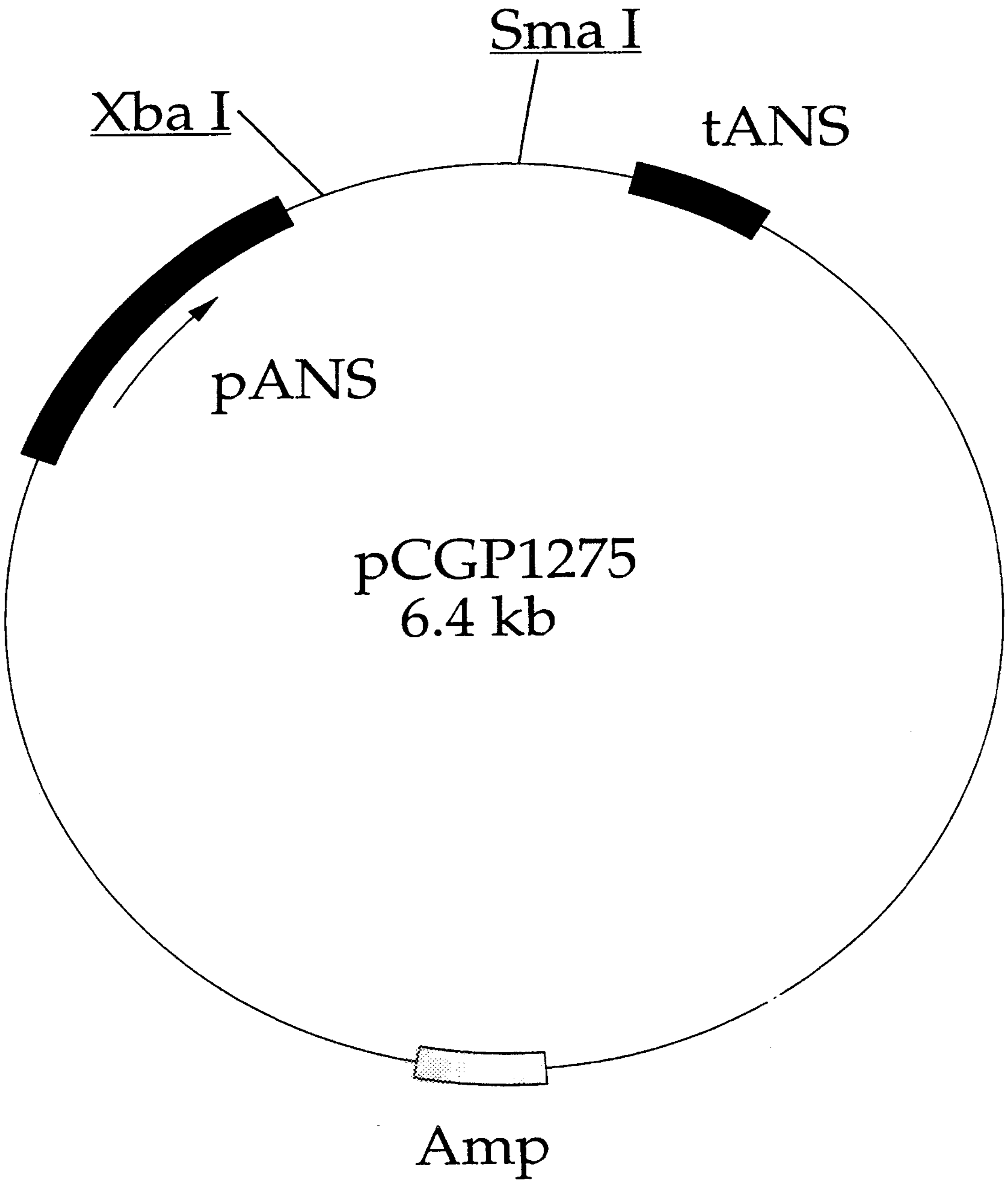


Figure 6d

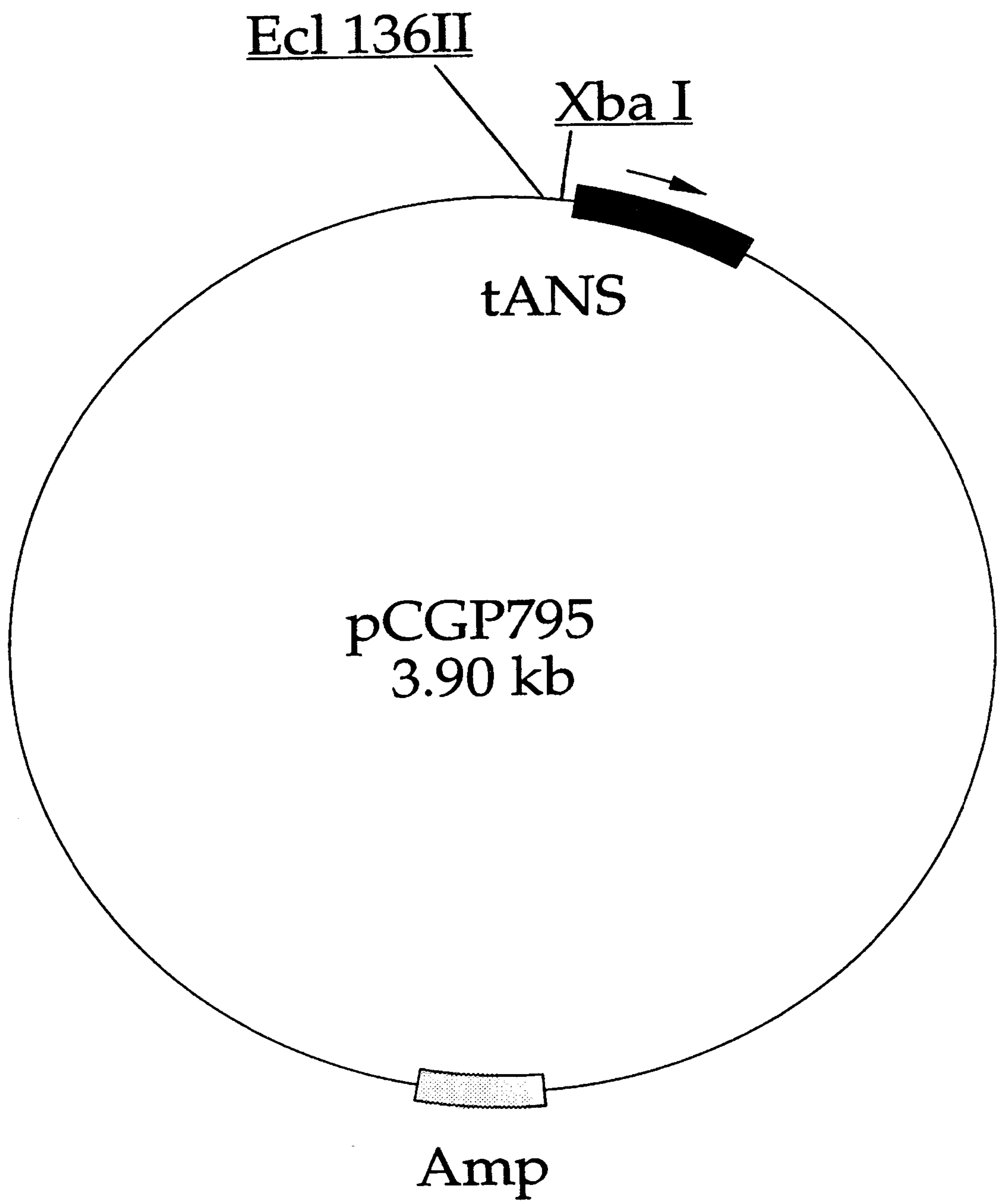


Figure 6e

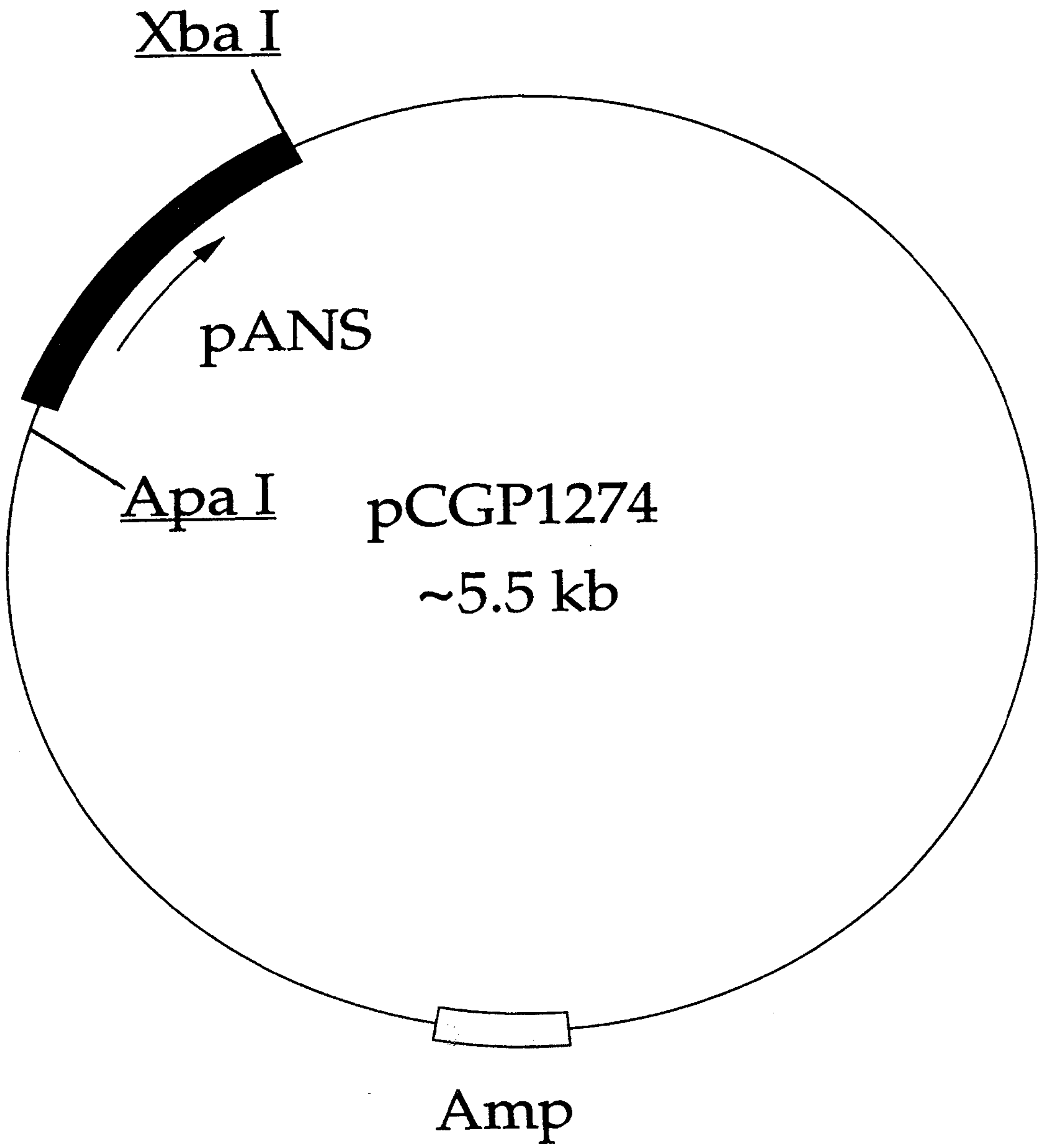
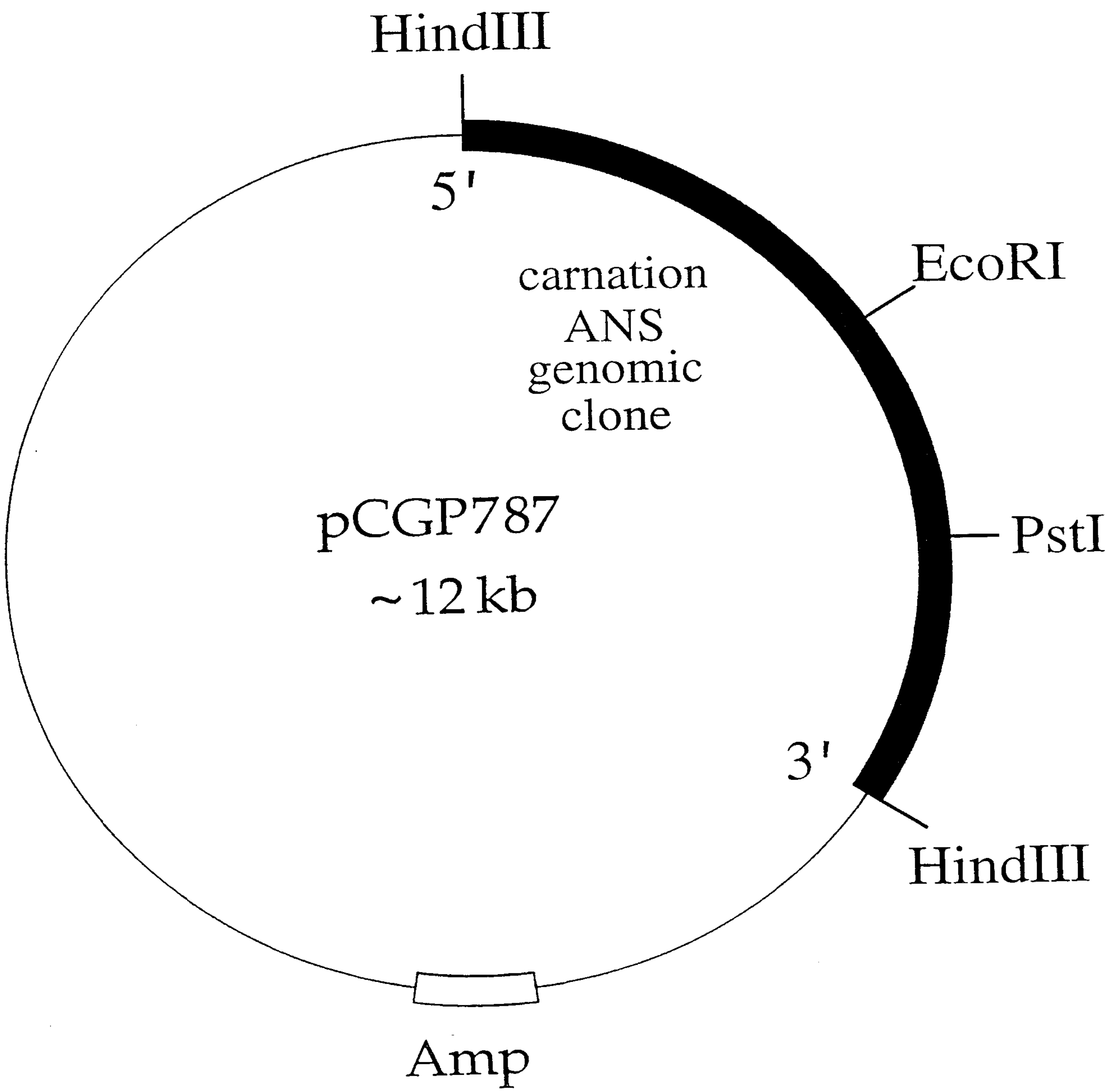


Figure 6f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carnation
ANS
genomic
clone
EcoRI
pCGP787
~12 kb
PstI
3'
HindIII
Amp

Figure 6g

Construct Schematic

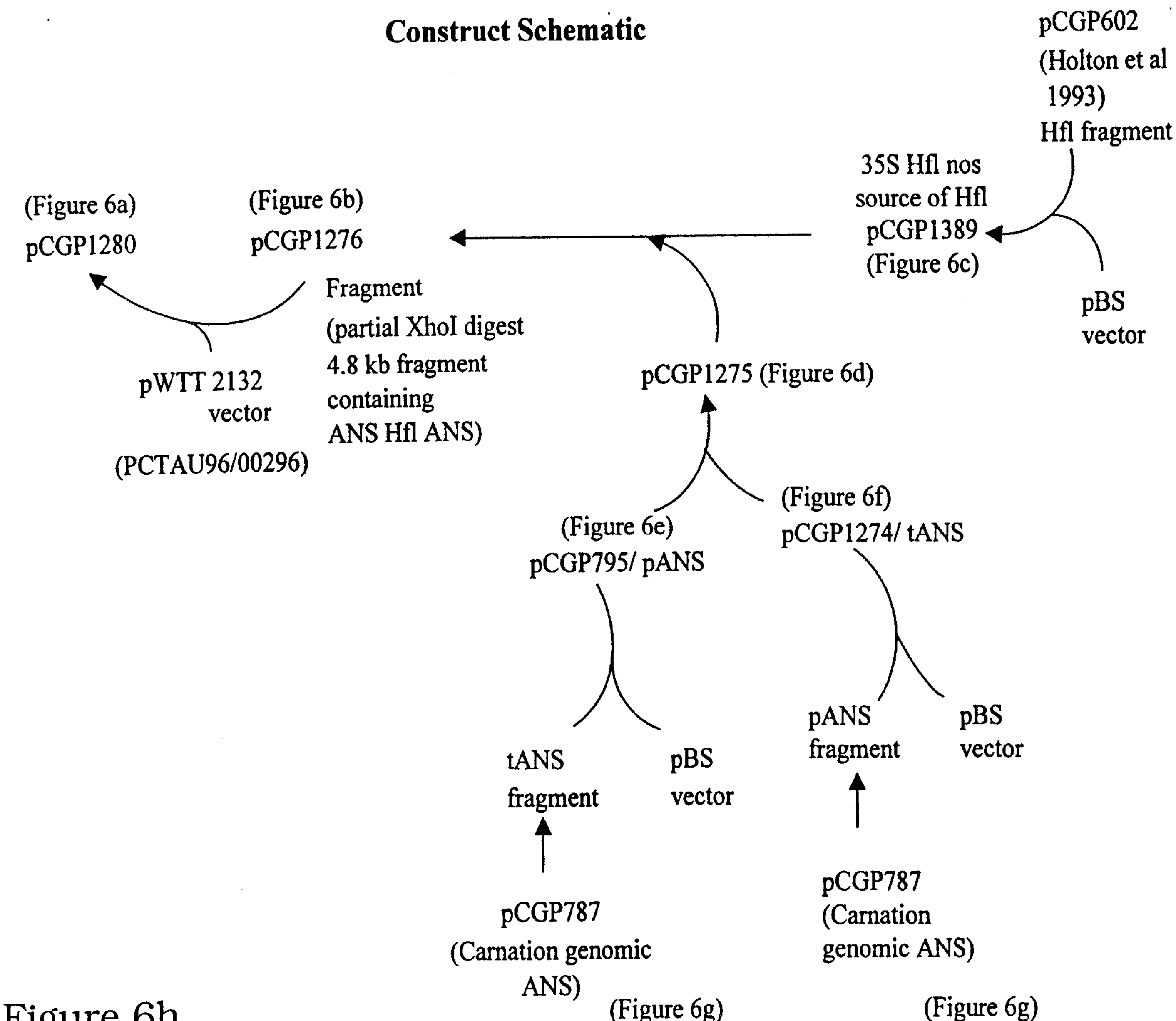
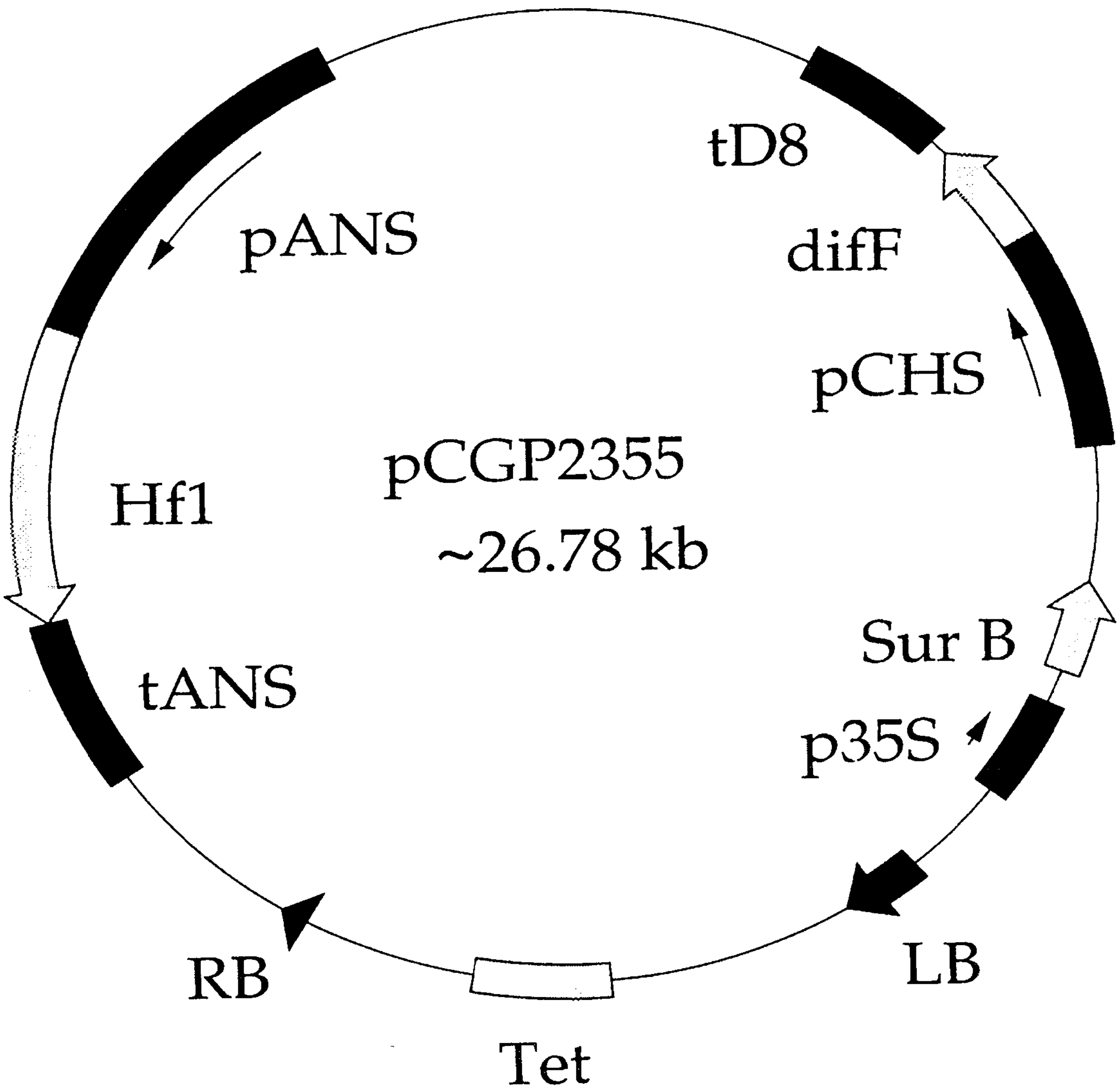


Figure 6h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Hf1
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~26.78 kb
tANS
Sur B
p35S
RB
LB
Tet

Figure 7a

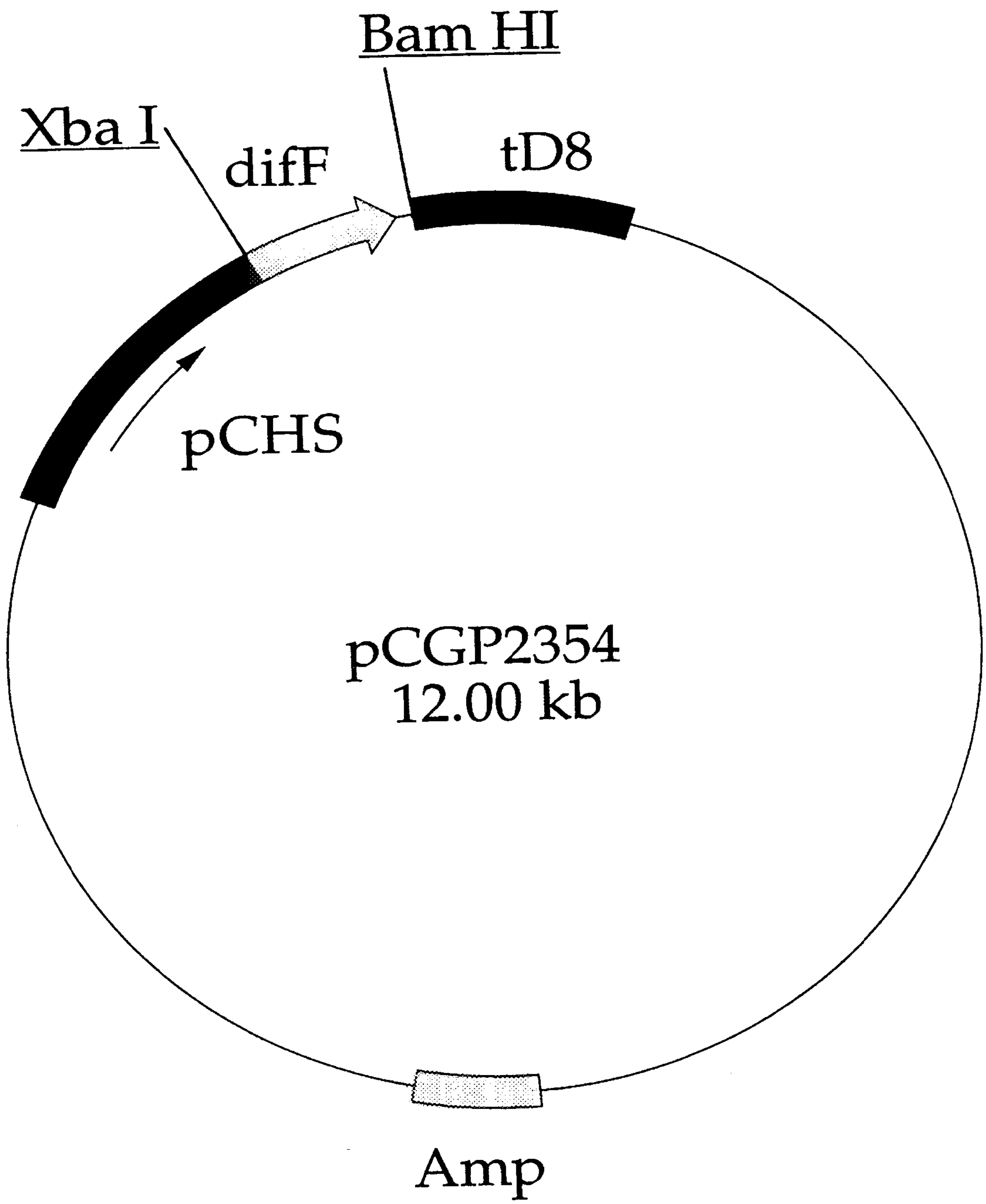


Figure 7b

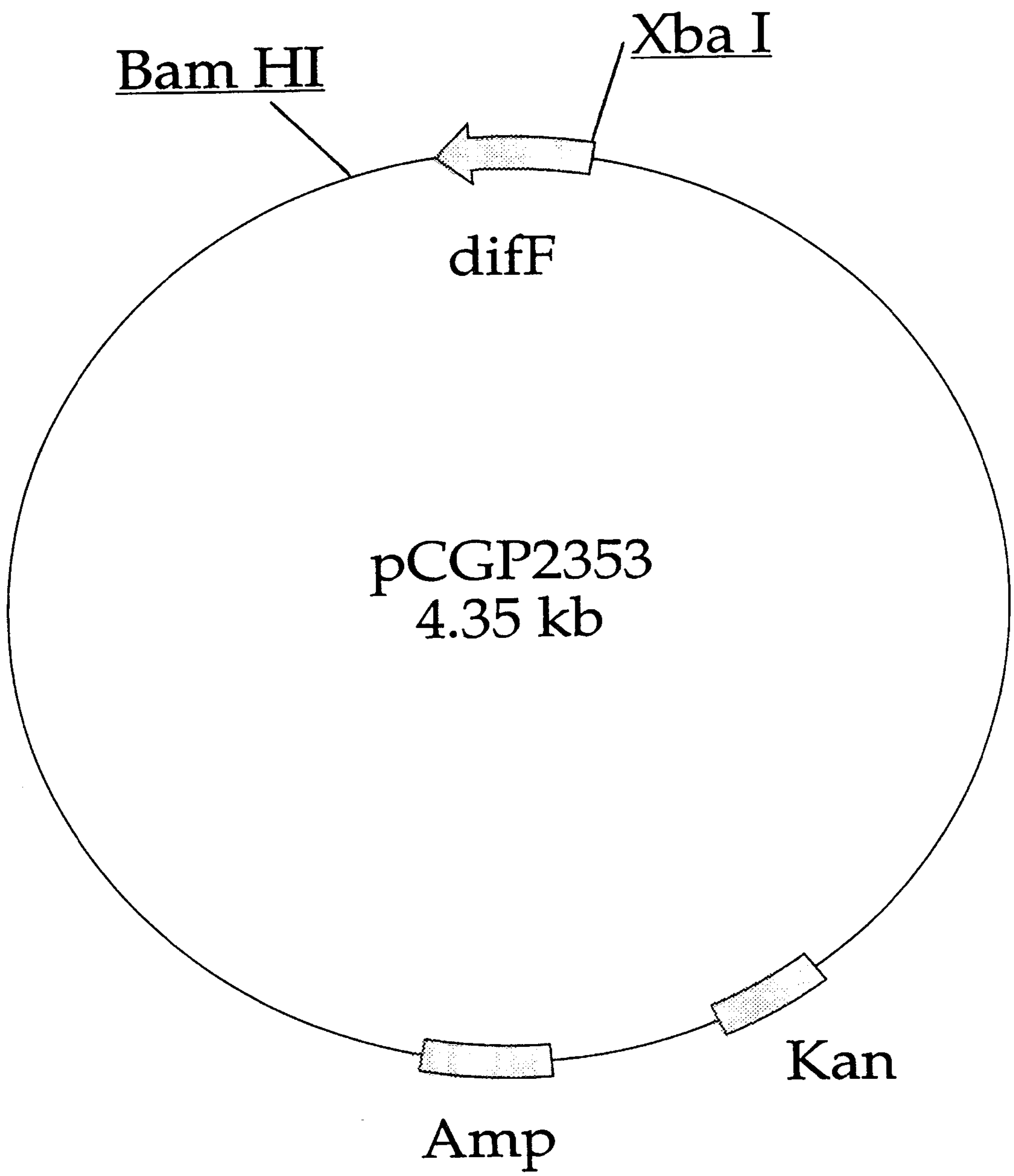


Figure 7c

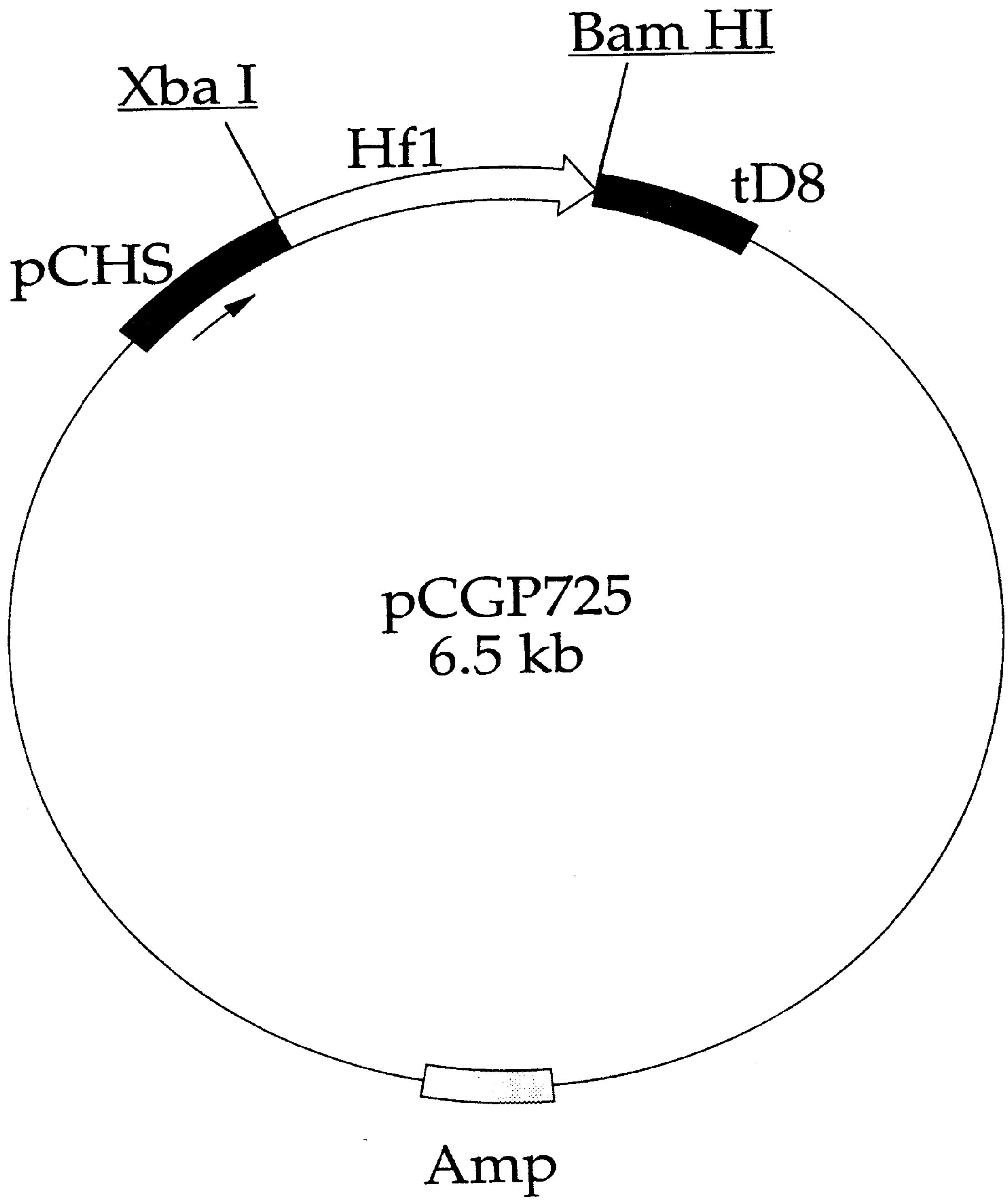
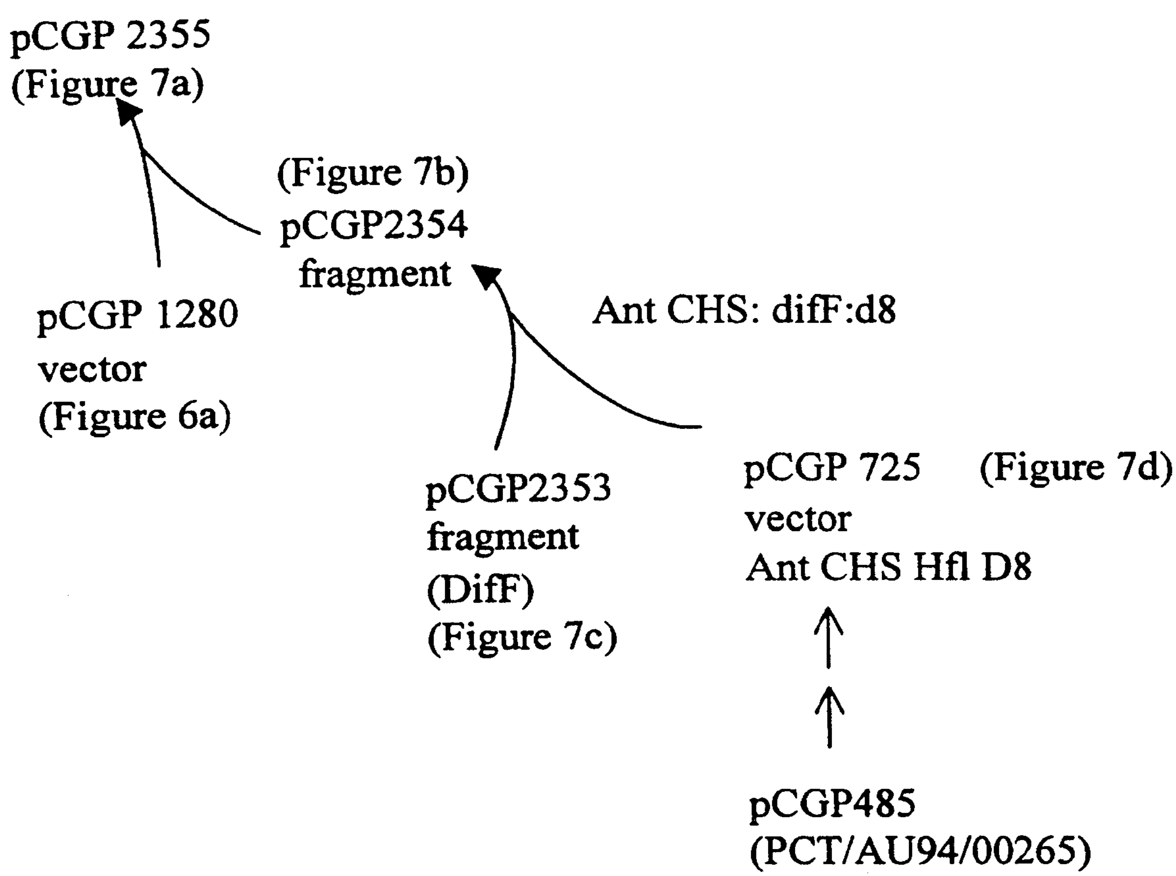


Figure 7d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(Figure 7a)
(Figure 7b)
pCGP2354
fragment
Ant CHS: difF:d8
pCGP 1280
vector
(Figure 6a)
pCGP2353
fragment
(DifF)
(Figure 7c)
pCGP 725 (Figure 7d)
vector
Ant CHS Hfl D8
pCGP485
(PCT/AU94/00265)

Figure 7e

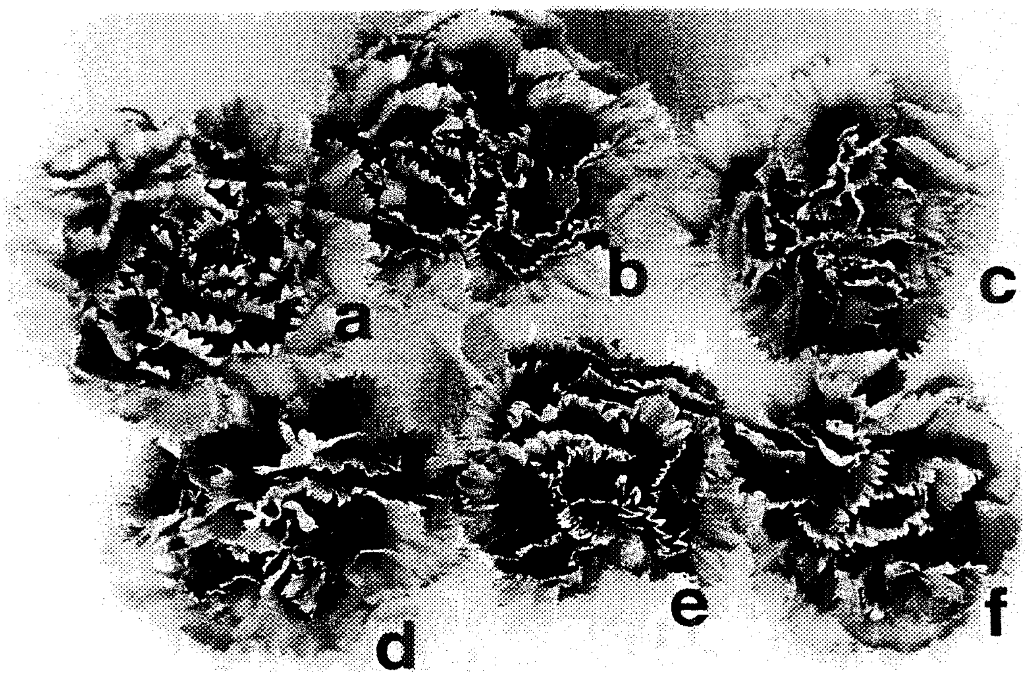
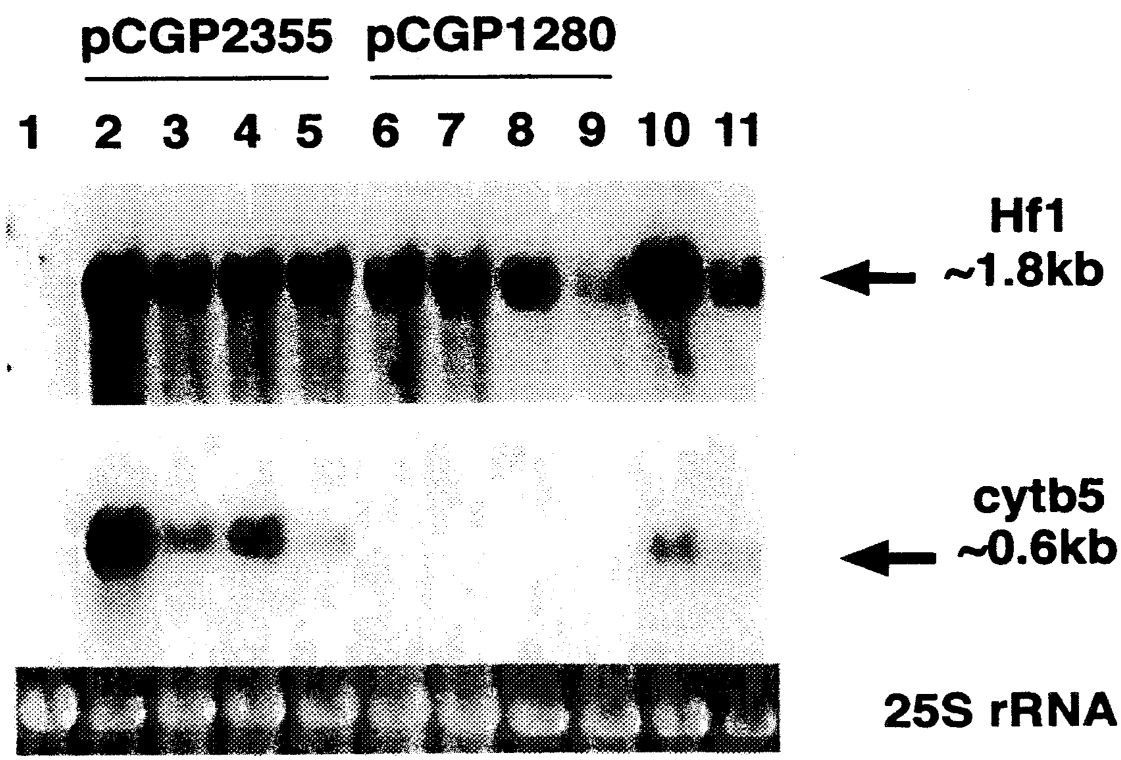


Figure 8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pCGP1280
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Hf1
~1.8kb
cytb5
~0.6kb
25S rRNA

Figure 9

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     1               5                  10                  15
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Lys Ser Lys Gln Asp Cys Trp Ile Ile Ile Asn Gly Arg Val Val Asp

20 25 30

gta aca aag ttc ttg gaa gaa cat cct gga gga gaa gaa gtg ttg att 143

Val Thr Lys Phe Leu Glu Glu His Pro Gly Gly Glu Glu Val Leu Ile

35 40 45

gaa tca gca gga aag gat gca act aaa gag ttt caa gat att gga cat 191

Glu Ser Ala Gly Lys Asp Ala Thr Lys Glu Phe Gln Asp Ile Gly His

50 55 60

agt aaa gct gcc aag aac ttg ctt ttc aaa tac caa att gga tat ctt 239

Ser Lys Ala Ala Lys Asn Leu Leu Phe Lys Tyr Gln Ile Gly Tyr Leu

65 70 75

caa ggt tac aaa gcc tca gat gat tct gaa ctt gaa ctc aac tta gtc 287

Gln Gly Tyr Lys Ala Ser Asp Asp Ser Glu Leu Glu Leu Asn Leu Val

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Thr Asp Ser Ile Lys Glu Pro Asn Lys Ala Lys Glu Met Lys Ala Tyr

100 105 110

gtt atc aaa gaa gat cct aag cca aag tat ctg act ttt gtt gag tac 383

Val Ile Lys Glu Asp Pro Lys Pro Lys Tyr Leu Thr Phe Val Glu Tyr

115 120 125

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Leu Leu Pro Phe Leu Ala Ala Ala Phe Tyr Leu Tyr Tyr Arg Tyr Leu
130 135 140

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Thr Gly Ala Leu Gln Phe
145

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35 40 45

Ser Ala Gly Lys Asp Ala Thr Lys Glu Phe Gln Asp Ile Gly His Ser
50 55 60

Lys Ala Ala Lys Asn Leu Leu Phe Lys Tyr Gln Ile Gly Tyr Leu Gln
65 70 75 80

Gly Tyr Lys Ala Ser Asp Asp Ser Glu Leu Glu Leu Asn Leu Val Thr
85 90 95

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100 105 110

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Thr Lys Phe Leu Glu Glu His Pro Gly Gly Glu Glu Val Leu Ile Glu
35 40 45

Ser Ala Gly Lys Asp Ala Thr Lys Glu Phe Gln Asp Ile Gly His Ser
50 55 60

Lys Ala Ala Lys Asn Leu Leu Phe Lys Tyr Gln Ile Gly Tyr Leu Gln
65 70 75 80

Gly Tyr Lys Ala Ser Asp Asp Ser Glu Leu Glu Leu Asn Leu Val Thr
85 90 95

Asp Ser Ile Lys Glu Pro Asn Lys Ala Lys Glu Met Lys Ala Tyr Val
100 105 110

Ile Lys Glu Asp Pro Lys Pro Lys Tyr Leu Thr Phe Val Glu Tyr Leu
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taggagtcga gatgcgaaat tgtcgtttgc gactgtatac cttgatagaa atggatgcat 660

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